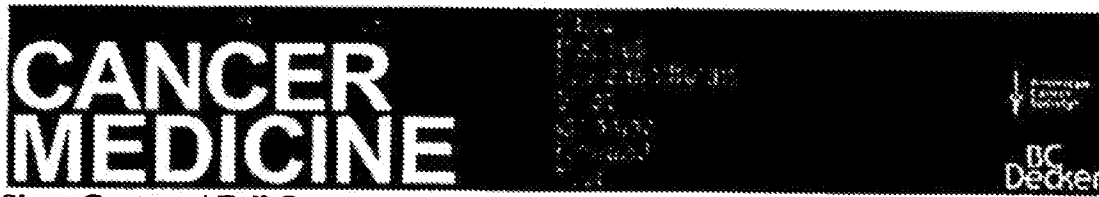


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Cancer Medicine → Part II Scientific Foundation, Section 1: Cancer Biology

11. Tumor Angiogenesis

Judah Folkman, MD
Raghu Kalluri, PhD

Angiogenesis, the growth of new capillary blood vessels, is central to the growth of cancer. An understanding of the cellular and molecular basis of tumor angiogenesis is therefore important for clinicians who diagnose and treat cancer by whatever modalities. This chapter is focused on certain general principles of tumor angiogenesis that are intrinsic to the behavior of human cancer. Almost every week, the biomedical literature contains elegant reports on the cause and development of cancer at the cellular and molecular levels. This rapid progress in understanding the molecular and genetic events that underlie the transformation of a normal cell to a cancer cell is reflected in many chapters in this book. These studies provide strong evidence for the hope that, eventually, there will be molecular solutions to the cancer problem. However, in virtually all scenarios of current or future therapy, the target is the cancer cell.

Experimental evidence indicates that it is prudent to develop cancer therapies against another target, the microvascular endothelial cell, without implying that the two targets are mutually exclusive.

Consider a cancer cell that has progressed through a series of mutations so that by activation of certain oncogenes and by loss of specific suppressor genes, it has become self-sufficient in growth signals, insensitive to antigrowth signals, unresponsive to apoptotic signals, capable of limitless replicative potential, and tumorigenic.¹ Are these neoplastic properties necessary and sufficient for such a cell to expand into a population that is clinically detectable, symptomatic, or lethal? Current evidence argues that these neoplastic properties may only be necessary but not sufficient for the cancer cell to become metastatic and lethal. The reported studies suggest that the microvascular endothelial cell dictates to a cancer cell whether it can grow a tumor to a clinically detectable size, metastasize, or kill its host. For a tumor to develop a metastatic or a lethal phenotype, it must first recruit and sustain its own private blood supply, a process called tumor angiogenesis.¹ Tumors unable to induce angiogenesis remain dormant at a microscopic in situ size. Such nonangiogenic lesions are usually not detectable unless they are on external surfaces such as skin, oral mucosa, or cervix. In immunodeficient mouse models, heterotransplanted malignant cells sometimes fail to form grossly-identifiable tumor nodules but nonetheless persist as small nonangiogenic

tumors called "no-takes." ²

Angiogenesis is fundamental to reproduction, development, and repair. In the adult, repair and reproductive angiogenesis occur mainly as brief bursts of capillary blood vessel growth that usually last only days or weeks. Such physiologic angiogenesis, including neovascularization in exercised muscle, is tightly regulated.³⁻⁵

A variety of circulating and sequestered inhibitors suppress proliferation of vascular endothelium under normal conditions. As a result, endothelial cells are among the most quiescent cells of the body. Turnover times of endothelial cells are measured in hundreds of days in contrast to bone marrow cells, which maintain an average turnover time of 5 days and proliferate at approximately 6 billion cell divisions per hour. During angiogenesis, microvascular endothelial cells can proliferate as rapidly as bone marrow cells. Furthermore, endothelial proliferation is not the only event necessary for development of a new capillary blood vessel. Endothelial cells must degrade their own basement membrane, develop sprouts from preexisting microvessels, invade the extracellular matrix, form tubes, and connect the tips of these tubes to create loops capable of handling blood flow.^{6, 7} Even in the absence of endothelial DNA synthesis in tissue that has been heavily irradiated, new capillary blood vessels and their branches still develop for a few days. ⁸

A hallmark of pathologic angiogenesis is persistent growth of blood vessels (ie, sustained neovascularization). Angiogenesis that continues for months or years supports the progression of many neoplastic and nonneoplastic diseases.^{9,10} However, both physiologic and pathologic angiogenesis are usually focal. An angiogenic focus appears as only a tiny fraction or a small "hot spot" of proliferating and migrating endothelial cells that arise from a monolayer of resting endothelium of approximately 1000 m², an area the size of a tennis court. A cubic millimeter of human cardiac muscle contains approximately 2,500 millimeters of capillary blood vessels, (as determined by stereologic methods).⁴ The fundamental objective of all antiangiogenic therapy is to return a pathologic neovascular focus to its normal resting state or to prevent its initiation.

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Beginning of angiogenesis research

Hypothesis: tumor growth depends on angiogenesis

In 1971, Folkman proposed a new view of the role of blood vessels in tumor growth in the form of a hypothesis that tumor growth is angiogenesis dependent.¹⁸ This hypothesis suggested that tumor cells and vascular endothelial cells within a neoplasm may constitute a highly integrated ecosystem and that endothelial cells may be switched from a resting state to a rapid growth phase by a "diffusible" chemical signal from tumor cells. An additional speculation was that angiogenesis could be a relevant target for tumor therapy (ie, antiangiogenic therapy). Folkman proposed these ideas from experiments he performed with Frederick Becker in the early 1960s, which revealed that tumor growth in isolated perfused organs was severely restricted in the absence of vascularization of the tumors.^{19–24}

These concepts were not accepted at the time. Although a few investigators in the early 1970s perceived that tumors could actually induce neovascularization, the belief persisted that such neovascularization was an inflammatory host response to necrotic tumor cells or possibly a host defense detrimental to the tumor. Another obstacle to research on tumor angiogenesis was the conventional wisdom at that time that any new vessels induced by a tumor, like new vessels in a wound, would become established and thus could not be made to involute. From this assumption, scientists concluded that antiangiogenic therapy could never regress a tumor; therefore, it would be fruitless to try to discover angiogenesis inhibitors. In this pessimistic atmosphere, it was not an easy task to produce compelling evidence that tumor growth depended on neovascularization. Eventual acceptance of the 1971 hypothesis was slow because it would be 2 more years before the first vascular endothelial cells were successfully cultured in vitro, 8 years before it was possible to grow capillary endothelial cells in vitro, 11 years before the discovery of the first angiogenesis inhibitor, and 13 years before the purification of the first angiogenic protein.^{25–29}

Throughout the 1970s, laboratory studies were devoted to demonstrating that: tumor vessels were new proliferating capillaries; the sequential steps of the angiogenic process could be identified; qualitative and quantitative bioassays for angiogenesis could be developed; viable tumor cells released diffusible angiogenic factors that stimulated new capillary growth and endothelial mitosis in vivo, despite the arrest of tumor cell proliferation by

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irradiation; necrotic tumor products were not angiogenic per se (reviewed in Folkman and Cotran); and that angiogenesis itself could be inhibited.³⁰⁻³⁵ Because of these efforts to provide supporting evidence that tumor growth was angiogenesis dependent, the field of angiogenesis research began. Today the field has broadened to include a wide spectrum of basic science disciplines, from developmental biology to molecular genetics, as well as a variety of clinical specialties, which include in addition to oncology, cardiology, dermatology, gynecology, ophthalmology, and rheumatology. Approximately 40 publications with angiogenesis in the title, appear every week. [↑ top](#)

Experimental evidence

By the mid-1980s, considerable experimental evidence had been assembled to support the hypothesis that tumor growth is angiogenesis dependent. The idea could now be stated in its simplest terms: "Once tumor take has occurred, every further increase in tumor cell population must be preceded by an increase in new capillaries that converge upon the tumor."³² The hypothesis predicted that if angiogenesis could be completely inhibited, tumors would become dormant at a small, possibly microscopic, size.²² It forecast that whereas the presence of neovascularization would be necessary, but not sufficient for expansion of a tumor, the absence of neovascularization would prevent expansion of a primary tumor mass beyond 1 to 2 mm³ and restrict a metastasis to a microscopic dormant lesion. Most nonneovascularized tumors are not clinically detectable, with the exception of surface lesions on the skin or the external mucous membranes.

The hypothesis that tumors are angiogenesis dependent is supported by biologic and pharmacologic evidence and proved by genetic evidence. Both types of evidence are summarized below because they provide a scientific basis for current clinical trials of angiogenesis inhibitors. [36, 37](#)

Biologic evidence

1. In two-dimensional flat cultures, a population of tumor cells expands indefinitely as long as fresh medium is added and unlimited cell-free surface is provided (ie, passage of cells to a new flask). In contrast, three-dimensional spheroids of the same cells, suspended in soft agar or methylcellulose, stop enlarging at a diameter of a few millimeters, despite repeated passage of the spheroids to fresh media.³⁸ In these "steady-state" spheroids, cell proliferation is balanced by cell death.³²⁻⁴¹ This in vitro model is analogous to a dormant micrometastasis in which angiogenesis is blocked.⁴²

2. Tumors implanted into subcutaneous transparent chambers grow slowly before vascularization, and tumor volume increases linearly. After vascularization, tumor growth is rapid and tumor volume may increase exponentially.^{15, 43}

3. Tumor growth in the avascular rabbit cornea proceeds slowly at a linear

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rate, but converts to exponential growth after neovascularization. ⁴⁴

4. Tumors suspended in the aqueous fluid of the anterior chamber of the rabbit eye remain in a dormant state: viable, avascular, and limited in size ($<1 \text{ mm}^3$). These tumors induce neovascularization of iris vessels, but the new vessels are out of reach of the tumors floating in the aqueous fluid. Once a tumor spheroid is apposed to the proliferating iris vessels, the tumor becomes neovascularized and can enlarge up to 16,000 times its original volume within 2 weeks. ⁴⁵ A current interpretation of this experiment is that the tumor spheroid had already switched to the angiogenic phenotype, but that angiogenesis was blocked by separation of the floating tumor from the nearest vascular bed. ⁴⁶

5. Tumors grown in the vitreous of the rabbit eye remain viable but are restricted to diameters of less than 0.50 mm for as long as 100 days. Once such a tumor reaches the retinal surface, it becomes neovascularized and within 2 weeks can undergo a 19,000-fold increase in volume over the avascular tumor. ⁴⁷ Cross-sectional histology of the avascular tumors reveals proliferating cells at the periphery of the tumor and necrotic tumor cells in its center.

6. Human retinoblastomas that have metastasized to the vitreous are viable and avascular and tumor expansion is restricted. ⁴⁸

7. Within a solid tumor, the $[\text{H}^3]$ thymidine labeling index of tumor cells decreases with increasing distance from the nearest open capillary vessel. ⁴⁹ The tissue oxygen tension also decreases with increasing distance of a tumor cell from the center of a capillary vessel. ⁵⁰ Because the limit of oxygen diffusion is 100 to 200 μm , tumor cells that exceed these distances from a capillary vessel become anoxic (as determined by infrared spectroscopy of tumors in transparent skin chambers in mice). ⁵¹

8. Tumors implanted into the chorioallantoic membrane of the chick embryo remain restricted in growth during the avascular phase, but enlarge rapidly once they are vascularized. ⁵²

9. Vascular casts of metastases in the rabbit liver reveal that tumors of up to 1 mm in diameter are usually avascular, but beyond that size are vascularized. ⁵³

10. Human ovarian carcinomas metastasize to the peritoneal membrane as tiny avascular seeds. These implants rarely grow beyond a limited diameter of a few millimeters, until after vascularization. This "avascular" state of peritoneal implants has also been demonstrated in mice with four different tumor types and is independent of whether the mice are immunocompetent or immunodeficient. The avascular peritoneal implants are less than 0.5 mm diameter and are of uniform size (C. Chen, personal communication, 1998).

11. In transgenic mice that develop carcinomas of the beta cells in the pancreatic islets, large tumors arise from a subset of preneoplastic

hyperplastic islets, but only after they have become vascularized. ⁵⁴

12. Neoplastic cells injected subcutaneously develop into tumors that become vascularized at about 0.4 mm³. ⁵⁵ As tumor size increases, blood vessels continue to proliferate and are enveloped by tumor cells that appear to grow toward and around the new vessels. The vessels eventually occupy up to 1.5% of the tumor volume. This is a 400% increase in vascular density over normal subcutaneous tissue. ⁵⁵ In this model, new microvascular sprouts that converge on a tumor are enveloped by tumor cells, giving the appearance that the tumor has been penetrated by vessels.

13. In colon carcinomas arising in rats after carcinogen exposure, there is an early phase (tumor diameter <3.5 mm) during which the tumor is temporarily supplied by preexisting host microvessels that are dilated and widened. ⁵⁶ This stage is similar to "cooption" of blood vessels recently reported. ⁵⁷ Subsequently, new capillary vessels sprout and proliferate (angiogenesis), which leads to increasing microvessel density and rapid tumor growth.

Pharmacologic evidence

By the late 1980s it became possible to inhibit tumor angiogenesis by biochemical and molecular methods. These experiments were based on (1) administration of molecules that inhibited angiogenesis specifically or selectively, (2) blockade of tumor-derived angiogenic factors, and (3) the development of spontaneous tumors in transgenic mice. Some examples of such are listed below.

1. An angiogenesis inhibitor, TNP-470 (AGM-1470), a synthetic analogue of fumagillin, selectively inhibited proliferating endothelial cells in vitro and in vivo. ⁵⁹ It potently inhibited tumor growth in vivo but did not inhibit tumor cells in vitro. In pre-clinical studies it has a broader spectrum of antitumor activity than any known anticancer drug. There are more than 60 reports from different laboratories of inhibition of 33 different types of human, mouse, rat, hamster, and rabbit primary tumors in animals and of 23 different types of metastatic tumors including human tumor metastases in mice and rat and hamster tumor metastases. Tumor growth was inhibited at an average of 65% with a range of 43 to 100% (ie, complete regression). All of these reports employed the same dose, 30 mg/kg every other day. Complete regression was observed in seven tumor types in mice, including neurofibrosarcoma, neurofibroma, breast cancer, gastric cancer, and choriocarcinoma, as well as mouse reticulum sarcoma and gastric carcinoma. ⁶⁰ Because TNP-470 inhibits microvascular endothelial cells at a concentration 3 logs lower than tumor cells, this angiogenesis inhibitor is virtually a specific inhibitor of endothelial proliferation and migration. These studies provided the first clue that angiogenesis inhibitors could be broad spectrum anticancer agents that are relatively independent of tumor type. The appearance of different therapeutic responses by different tumor types (eg, 43 to 100%) was more adequately explained by the total angiogenic output of each tumor type matched against a fixed dose of angiogenesis inhibitor. (See below for improvements in TNP-470.)

2. In another experiment, antibody against bFGF (basic fibroblast growth factor) secreted by a tumor administered to mice bearing the tumor, resulted in dramatic reduction in neovascularization and in tumor volume.

⁶¹ To engineer bFGF that was secreted from the tumor, the cDNA for human bFGF hybridized to a signal sequence was transfected into normal mouse fibroblasts. ⁶¹ Furthermore, the structure of the bFGF was modified by site-specific mutagenesis in which two serines were substituted for cysteines. Thus, the bFGF released by the tumor could be neutralized by a specific antibody that had no effect on natural bFGF. The transfected fibroblasts became tumorigenic, exported bFGF, and were highly angiogenic. They formed large lethal tumors when implanted into mice. Tumor angiogenesis was mediated solely by bFGF released from these tumors.

3. In an analogous experiment, a neutralizing antibody to another angiogenic protein, vascular endothelial growth factor (VEGF), was administered to mice bearing tumors that induced angiogenesis solely by VEGF. ⁶² Tumor growth was inhibited by more than 90%. The antibody had no effect on the tumor cells in vitro. With but few exceptions, VEGF is considered to be a specific mitogen for vascular endothelial cells. ⁶³

4. Specific immunologic inhibition of overexpression of the integrin $\alpha_v\beta_3$ on capillary endothelial cells resulted in apoptosis of proliferating endothelial cells, blocked neovascularization, and induced tumor regression. ⁶⁴

5. Endogenous specific inhibitors of endothelial proliferation and of angiogenesis include: angiostatin, a 38 kDa internal fragment of plasminogen (generated by Lewis lung carcinoma), endostatin, a 20 kDa internal fragment of collagen XVIII (generated from a murine hemangioendothelioma), a 53 kDa conformationally changed fragment of antithrombin III (generated from human small cell lung cancer), and tumstatin, a 28 kDa internal fragment of collagen IV (α_3 NC1 domain). ⁶⁵⁻⁷¹ They do not inhibit proliferation of resting confluent endothelial cells, epithelial cells, smoothmuscle cells, fibroblasts, or tumor cells in vitro. These proteins inhibit angiogenesis in the chick chorioallantoic membrane or in the mouse cornea. Both primary and metastatic tumors are markedly inhibited and tumor regression has been achieved without toxicity or drug resistance. ⁶⁹

6. In SCID mice bearing human pancreatic cancer, human recombinant endostatin was injected into the peritoneal cavity once daily, or delivered continuously by an implanted micro-osmotic pump that released 1 microliter/hour. ⁷² Continuous dosing inhibited tumor growth 10-fold more effectively than once a day bolus dosing and also induced tumor regression, whereas bolus dosing did not (Figure 11-1).

7. An antibody to two receptors of VEGF called VEGF-Trap, inhibits growth of VEGF producing tumors in mice. ⁷³

Genetic evidence

Genetic evidence that tumors are angiogenesis dependent began to be reported by the mid-1990s and provides the most compelling proof that tumors and their metastases cannot not grow beyond a microscopic in situ size without recruiting new microvessels. These experiments are based on (1) transfection of dominant-negative receptors for a proangiogenic protein into endothelial cells in the tumor bed, (2) transfection of genes for antiangiogenic proteins into tumor cells, (3) regulation of the expression of angiogenic oncogenes, and (4) manipulation of developmental genes. Some examples are listed below.

1. The growth of brain tumors in nude mice was significantly inhibited or prevented when tumor angiogenesis was suppressed by a strategy in which a dominant-negative mutant of the receptor (Flk-1) for the angiogenic protein VEGF was introduced into host endothelial cells (carried by a retrovirus). This signaling-defective receptor mutant formed an inactive dimer with the native Flk-1 receptor on endothelial cells and prevented the formation of new capillary blood vessels in response to VEGF released by the tumor. ⁷⁴

2. Transformed cells were not tumorigenic until after they had become angiogenic by downregulating expression of thrombospondin-1 a process that was p53-dependent. ⁷⁵

3. Transfection of normal human cells with the SV40 large T oncogene, generated immortalized cells in vitro. However, when these cells were inoculated into mice they formed microscopic sized nonangiogenic tumors that did not grow beyond approximately 1 mm diameter. ⁷⁶ A subsequent transfection with the *ras* oncogene switched the cells to the angiogenic phenotype, upregulated VEGF production, downregulated production of tissue inhibitors of metalloproteinases, and generated highly neovascularized, rapidly growing tumors.

4. Downregulation of the *ras* oncogene in a melanoma driven by doxycycline inducible *ras*, led to massive apoptosis of microvascular endothelium in the tumor bed starting within 6 hours. Tumor cells began to die days later, and large tumors had completely disappeared by 12 days. ⁷⁷

5. When tumor cells were transfected with the cDNA for angiostatin and implanted into mice, the higher the secretion of angiostatin, the slower the growth of tumors. However, tumor cell proliferation in vitro was not affected by angiostatin transfection. ⁷⁸

6. When tumors were transfected with the secretable antiangiogenic protein thrombospondin-1 (TSP-1) and/or thrombospondin-2 (TSP-2), tumor growth was directly proportional to suppression of angiogenesis. Tumor growth was inhibited up to 100% (ie, microscopic dormant tumor or no tumor) when angiogenesis was suppressed completely. Tumor cell proliferation was independent of level of TSP-1 or TSP-2 production. ⁷⁹

7. Id1 and Id3 are helix-loop-helix proteins that may control differentiation by interfering with DNA binding of transcription factors. After targeted

disruption of one allele of Id1 and two alleles of Id3, three different types of implanted tumors failed to induce angiogenesis, their growth was severely restricted, and they did not metastasize.⁸⁰ However, when these mice were injected with bone marrow from wild-type mice containing normal Id1 and Id3 markers, progenitor endothelial cells expressing Id1 and Id3 circulated from the bone marrow to the tumor vascular bed and permitted the tumors to undergo neovascularization and to grow.⁸¹



Metastasis is also angiogenesis dependent

Experimental and clinical evidence suggests that the process of metastasis is also angiogenesis dependent. For a tumor cell to metastasize successfully, it must breach several barriers and respond to specific growth factors.⁸²⁻⁸⁶ Thus, tumor cells must gain access to the vasculature in the primary tumor, survive the circulation, arrest in the microvasculature of the target organ exit from this vasculature, grow in the target organ, and induce angiogenesis.⁸⁴⁻⁹⁰ Therefore, angiogenesis appears to be necessary at the beginning as well as at the completion of the metastatic cascade.

In experimental animals, tumor cells are rarely shed into the circulation before a primary tumor is vascularized, but they can appear in the circulation continuously after neovascularization.^{91, 92} The number of cells shed from the primary tumor appears to correlate with the density of tumor blood vessels as well as with the number of lung metastases observed later. Tumor cells can enter the circulation by penetrating through proliferating capillaries that have fragmented basement membranes and are leaky.^{92, 93} Further, angiogenic factors from tumors such as bFGF and VPF/VEGF induce increased production of plasminogen activator and collagenases in proliferating endothelial cells, thus further contributing to degradation of basement membranes.⁹⁴⁻⁹⁶ These degradative enzymes may facilitate the entry of tumor cells into the circulation. Tumor cells may not immediately become neovascularized after reaching the target organ. Such a metastasis lacking angiogenic activity for a variety of reasons may remain as a microscopic tumor of 100 to 200 μm diameter indefinitely.^{42, 97, 98} It has been the conventional wisdom that in human dormant micrometastases (eg, metastases appearing 5 to 10 years after removal of a breast cancer), tumor cells are not cycling or are in G_0 . However, increasing experimental evidence, indicates that micrometastases can be held dormant by blocked angiogenesis that results in a balance of tumor cell proliferation and apoptosis.^{42, 97} Finally, experimental metastases are as susceptible as primary tumors to control by specific angiogenesis inhibitors.^{65, 66} However, the relative efficacy of a given angiogenesis inhibitor can be affected by differences in metastatic sites and by the presence or absence of the primary tumor.⁶⁵ \uparrow TOP

Prevascular phase limits tumor expansion

During the prevascular phase, when angiogenic activity is absent or insufficient, tumors remain small, with volumes measured in a few cubic millimeters. Growth of the whole tumor is slow, and doubling times for

the whole tumor may be years. However, this does not mean that the tumor cells are proliferating slowly. Experimental studies show that tumor cells in a prevascular neoplasm may have a [^3H]thymidine labeling index as high as that of a large vascularized tumor. The difference between the two is that the prevascular tumor reaches a steady state in which generation of new tumor cells is balanced by tumor cell apoptosis. ⁹²

When the prevascular phase of bladder cancer, cervical cancer, or cutaneous melanoma is first detected, these lesions are usually thin, slowly growing, stable for months to years, asymptomatic, and rarely metastatic. ¹⁰⁰⁻¹⁰³ For the majority of tumors, however, the prevascular stage is clinically undetectable and can only be observed microscopically. For example, in breast and prostate cancer, carcinomas in situ can be observed before and after neovascularization in the same specimen. ^{89, 104, 105}

The size limits of experimental tumors when angiogenesis is blocked or absent are between approximately 0.2 mm diameter (eg, for lung metastases in mice) and 2 mm (eg, for chondrosarcomas in rats, ¹⁰⁶ having a tumor population of 10^5 - 10^6 cells). ^{42, 106} The differences in size of prevascular (nonangiogenic) tumors may be due in part to the capacity of tumor cells to survive under differing degrees of hypoxia (Figure 11-2). ^{51, 107} However, if cancer cells are already angiogenic at the time of implantation, they can initiate angiogenesis before the tumor population would have reached the limiting size of a nonneovascularized tumor (ie, 0.2 to 2 mm). The evidence for this conclusion is from an experiment in which only 20 to 30 angiogenic mammary tumor cells were implanted into a transparent skin chamber in a mouse. ^{108, 109} The tumor cells elongated by day 2, migrated toward the nearest microvessels in a parallel orientation by day 4, divided unidirectionally, induced vascular dilation and tortuosity in the neighboring microvessels by day 6, and stimulated new vascular sprouts and loops with intermittent blood flow by day 8. By this time, a microscopic colony of up to 300 to 400 cells was visible. Tumor cells preferentially grew contiguous to the microvessel sprouts, and by day 20 the tumor was filled with a newly formed vasculature. When angiogenesis was prevented by injection into the chamber of a truncated soluble receptor (Flk 1) for vascular endothelial growth factor (VEGF), some tumor cells underwent apoptosis and microscopic tumors regressed or became dormant within 5 days (ie, before the appearance of neovascular sprouts). The soluble receptor potently inhibited endothelial cell proliferation in vitro, but had no effect on tumor cells. This result provides evidence for the operation of a two-way paracrine exchange of growth factors and survival factors between tumor cells and neighboring vascular endothelial cells (see below). In this model, tumor cells secrete angiogenic proteins that activate endothelial cells to elaborate chemoattractants for tumor cells. Simultaneously, the activated endothelium forms new vascular sprouts directed toward the tumor. Vascular endothelial cells can produce at least 20 mitogens and antiapoptotic factors. ¹¹⁰ Many of these proteins, such as basic fibroblast growth factor (bFGF) and heparin-binding epithelial growth factor (HB-EGF), are stored in the extracellular matrix and could be mobilized by VEGF stimulation of endothelial cells to secrete proteases. ¹⁰⁶

It is also possible that VEGF elaborated from tumor cells increases the permeability of local microvessels so that the tumor microcolony is bathed in nutrients even before neovascularization begins. The diffusion of nutrients (and oxygen) into the tumor bed would also be facilitated by loss of pericytes from microvessels due to endothelial elaboration of angiopoietin-2.¹¹¹ Endothelial cells up-regulate expression of angiopoietin-2 in the presence of tumor cells. Further, plasma and fibrin leakage from the microvessels could facilitate chemotaxis and alignment of tumor cells, as well as subsequent migration of endothelial cells.^{112, 113} VEGF also up-regulates synthesis of nitric oxide in vascular endothelium.^{114–116} Vasodilation and vessel tortuosity permit endothelial elongation and thus may be a prerequisite for endothelial cells to undergo mitosis and migration in response to angiogenic factors like VEGF.¹¹⁷ Even highly transformed neoplastic cells respond more efficiently to mitogens when the cells are elongated or spread than when they are rounded.^{118, 119} Furthermore, when well-vascularized tumors in mice were treated with antiVEGF monoclonal antibodies, there was a dramatic reduction in diameter, tortuosity, and vascular permeability in the tumor vessels.⁵⁸

In summary, when a microscopic population of tumor cells emerges in avascular epidermis or mucosa-before becoming angiogenic-it may remain dormant as an in situ carcinoma separated from its vascular bed by a basement membrane.⁴⁶ If a nonangiogenic tumor emerges in vascularized tissue (ie, an islet cell carcinoma) or as a micrometastasis, it may form a microcylinder of tumor cells around preexisting capillary vessels (also called cooption).^{42, 46, 57, 58, 98} In all of these situations, the pre-angiogenic tumor is restricted in size to a range of approximately 0.2 mm to 2 mm diameter.^{42, 106} In contrast, a microscopic population of angiogenic tumor cells may begin to modify microvessels in the neighborhood by inducing an increase in vascular permeability, dilation, and tortuosity even before the induction of new vascular sprouts.⁵⁶ [↑ top](#)

Human tumors and spontaneous animal tumors switch to an angiogenic phenotype

Mechanisms of the angiogenic switch

Normal cells that have been transformed to neoplastic cells are not usually angiogenic at the outset. Experimental studies of spontaneous tumors in transgenic mice reveal that the angiogenic switch is a discrete event that develops during progressive stages of tumorigenesis, beginning with the premalignant stage in these mouse models.^{46, 120, 121} By the time most human tumors are detected, for example, by a positive mammogram, neovascularization has usually occurred. However, most human tumors arise without angiogenic activity, exist in situ without neovascularization for months to years, and then switch to an angiogenic phenotype.¹²¹ Therefore, the angiogenic phenotype appears after the expression of the malignant phenotype in the majority of primary tumors. However, for certain human tumors such as carcinoma of the cervix, the preneoplastic stage of dysplasia becomes neovascularized before the malignant tumor

appears.¹⁰² This sequence of events also occurs in certain spontaneously arising tumors in animals.

At least four mechanisms of the angiogenic switch have been identified in both human tumors and spontaneous tumors in mice ([Table 11-1](#)).

Prevascular tumors recruit their own blood supply

This is the most common mechanism of the angiogenic switch. Approximately 95% of human cancers are carcinomas that originate as microscopic in situ lesions in an avascular epithelial layer separated by a basement membrane from underlying vasculature in the dermis or submucosa, respectively. In microscopic ductules of breast or prostate containing in situ carcinoma that has switched to the angiogenic phenotype, one can observe a ring of new microvessels encircling the ductule. However, these vessels remain temporarily separated from the tumor cells by intact basement membrane. The basement membrane is a physical and molecular barrier to migrating endothelial cells. For example, tumstatin, a potent inhibitor of endothelial cell migration and proliferation is found in collagen IV.⁷¹ After the basement membrane has been breached by new vessel sprouts, tumor cells form multiple cell layers around each new capillary blood vessel.¹²² The radius of these microcylinders is restricted to the oxygen diffusion limit for a given tumor type as originally defined by Thomlinson and Gray.^{37, 50} For example, for a human melanoma, the oxygen diffusion limit is approximately 85 μm . Beyond that distance from a capillary blood vessel, virtually all tumor cells are apoptotic (or necrotic). Within that radius, most tumor cells are viable (see [Figure 11-2](#)). For a prostate carcinoma the oxygen diffusion limit is approximately 110 μm , and it may be greater for certain tumors, such as for a chondrosarcoma or a tumor in which p53 is mutated or absent, but rarely would 200 μm be exceeded. [↑ TOP](#)

Circulating endothelial stem cells in tumor angiogenesis

Recent experimental and clinical evidence reveals that circulating endothelial progenitor cells derived from stem cell reservoirs in the postnatal bone marrow can be recruited to the vascular bed of tumors and contribute to tumor growth.^{81,123–135} Functional vascular endothelial growth factor receptor-1 (VEGFR-1) is expressed on hematopoietic stem cells (CD34⁺ in humans and Lin[−]Sca-1⁺c-Kit⁺ in mice).¹³³ Functional VEGFR-2 is expressed on CD34⁺ AC133⁺ progenitor human endothelial cells. VEGF elaborated by a variety of tumor signals through both VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), can mobilize progenitor endothelial cells into the circulation where they are recruited into the vascular bed of certain tumor types, but not others.⁸¹

Before endothelial progenitor cells can leave the bone marrow, they must transfer (along with hematopoietic stem cells) from the quiescent to the proliferative niche within the marrow. Mobilization of hematopoietic stem cells and progenitor endothelial cells necessary for their transfer to the proliferative niche is dependent upon release of soluble Kit-ligand, which

itself depends upon upregulation of metalloproteinase-9.¹³⁴ Thus, the microvascular endothelial cells in the vascular bed of a tumor may be recruited both from the local neighborhood as well as from the bone marrow. Rafii has shown that the ratio of these endothelial cells from different sources may differ by tumor type (S. Rafii, personal communication,). For example, in lymphomas the majority (>90%) of endothelial cells are derived from the bone marrow. In breast cancer, bone marrow-derived endothelial cells may approximate the number of endothelial cells from the local neighborhood. In contrast, in prostate cancer the majority of endothelial cells are recruited locally and very few come from the bone marrow. At this writing, the ratios of bone marrow-derived endothelium and local endothelium have not been elucidated for a wide variety of tumors. Nevertheless, the possible implications of these studies are provocative.

First, the efficacy of conventional chemotherapy may turn out to correlate with the percent of bone marrow-derived endothelial cells in a tumor. Second, during conventional chemotherapy, the off therapy intervals necessary to rescue bone marrow may result in a surge of progenitor endothelial cells that could traffic to the tumor (S. Rafii, personal communication, 2002). Third, certain angiogenesis inhibitors may suppress the release of bone marrow-derived progenitor endothelial cells. Angiostatin targets progenitor endothelial cells; endostatin induces apoptosis in circulating endothelial cells in tumor-bearing mice, and thalidomide decreases circulating endothelial cells by 10-fold in multiple myeloma.¹³⁶⁻¹³⁸ Endothelial cells (P1H1-12) in the circulation may be shed from the vascular bed of a tumor, especially after therapy when apoptotic endothelial cells may appear in the circulation. Some of these cells may be apoptotic (J. Heymach and S. Soker, unpublished data). Quantification of these two populations of circulating endothelial cells, bone marrow-derived progenitor endothelial cells and endothelial cells shed from the vascular bed of a tumor, is currently being evaluated as a potential surrogate marker for efficacy of antiangiogenic therapy, or perhaps for chemotherapy.

Circulating VEGF may be one of the angiogenic signals by which tumors can recruit endothelial cells from bone marrow. Subcutaneously implanted collagen gels embedded with VEGF are invaded by endothelial cells from bone marrow as well as from the local neighborhood.⁸¹ VEGF serum concentrations closely correlate with platelet counts in cancer patients. VEGF is stored, transported, and released from platelets.^{355, 356} Furthermore, Pinedo and colleagues report that platelet counts have prognostic significance for cancer patients: higher platelet counts correlate with a worse prognosis.³⁵⁷⁻³⁵⁹ Therefore, it is possible that for those types of tumors that recruit bone marrow-derived endothelial cells, communication from tumor to bone marrow may be mediated in part by the VEGF in circulating platelets. At this writing, these results have as yet no direct impact on clinical cancer therapy. However, oncologists should be aware of a potential role for platelets in cancer growth, and in tumor angiogenesis. For example, bone marrow-supportive agents, currently used in high-dose chemotherapy, contribute to platelet production and thereby may influence response to therapy.³⁵⁷ ↑ TOP

Nonendothelial host cells may amplify tumor angiogenesis

In addition to recruiting vascular endothelium from the host, certain tumors may also attract mast cells, macrophages, and inflammatory cells.^{139–142} These cells can amplify tumor angiogenesis by releasing proangiogenic molecules such as bFGF, or by releasing metalloproteinases that can mobilize VEGF and other angiogenic proteins.^{106, 113, 139, 143} Tumor angiogenesis and tumor growth are significantly diminished in mice deficient in metalloproteinase-9.¹⁴¹ Certain tumor cells may also trigger host stromal cells in the tumor bed to overexpress the angiogenic protein VEGF.¹⁴⁴ This is another mechanism of amplification of the angiogenic phenotype once it has been initiated. [↑ top](#)

Vessel cooption

In certain metastases (eg, the mouse brain), it has been shown that tumor cells exit from microvessels in the target organ, begin to grow around these vessels, cause the endothelial cells to undergo apoptosis, and finally induce neovascular sprouts from neighboring vessels. This process, called "cooption," may represent an intermediate or alternative step in the switch to the angiogenic phenotype.^{57, 58}

The general hypothesis that tumors are angiogenesis dependent and that antiangiogenic therapy is a method of controlling tumor growth operates for any or all of these mechanisms of angiogenic switching. [↑ top](#)

Persistence of nonangiogenic tumor cells in the vascularized tumor

After a nonangiogenic microscopic in situ carcinoma has become neovascularized, a significant percentage of tumor cells remain nonangiogenic.² The vascularized human primary tumors that we have studied in the Folkman lab so far, generally appear to contain a mixture of angiogenic and nonangiogenic tumor cells. The nonangiogenic tumor cells can be isolated from a human tumor removed at surgery by implanting numerous tiny pieces (1 mm³) of tumor into the subcutaneous dorsum of immunodeficient SCID mice, or by implanting cultured human tumor cells.² While a few tumors grow to a palpable and visible size (100 mm³ to >1,000 mm³) within a few weeks, and a few others appear after several months, most human tumor transplants remain invisible in the majority of mice, a phenomenon called "no take." However, upon opening the skin of these mice, one invariably finds a tiny (<0.5 to 1 mm³) whitish avascular or poorly vascularized tumor that is transplantable, contains proliferating and apoptotic tumor cells, but does not expand nor metastasize. A few of these nonangiogenic dormant tumors have spontaneously become angiogenic after 1 or more months, depending on the tumor type and have then grown. Others (eg, osteosarcoma) have remained nonangiogenic and dormant up to 3 years after being transplanted to new mice every 8 months (Figure 11-3).^{2, 22} Some tumor types that remain nonangiogenic can be rapidly switched to the angiogenic phenotype by transfection with the ras oncogene, which significantly increases tumor cell production of VEGF

and decreases thrombospondin-1 production.⁹² Labeling the nonangiogenic tumor cells by transfection with green fluorescent protein (GFP), permits them to be visualized beneath the skin. When the green nonangiogenic tumor cells are mixed 1:1 with angiogenic tumor cells that are not labeled, or mixed with angiogenic tumor cells transfected with red fluorescent protein (T. Udagawa et al unpublished data), the resulting large neovascularized tumor, which grows up rapidly, contains tiny (<0.5 mm) green nonangiogenic colonies dispersed throughout.⁹² When the nonangiogenic green tumor cells are mixed with a decreasing fraction of angiogenic tumor cells, there is a long latent period of one or more months before neovascularized tumors arise. The latent period appears to be the time required for the angiogenic tumor cells to accumulate to a threshold population sufficient to recruit new blood vessels for the whole tumor. Once neovascularization has occurred, spontaneous metastases may appear in the lung (depending on tumor type). Non-angiogenic tumor cells form microscopic nonangiogenic metastases (T. Udagawa et al, unpublished personal communication). These studies indicate that a nonangiogenic, dormant primary tumor may not shed tumor cells into the circulation. Once it switches to the angiogenic phenotype, it may then shed both angiogenic and nonangiogenic tumor cells into the circulation. The nonangiogenic tumor cells may be the source of microscopic metastases that are capable of remaining dormant for prolonged periods. It has also been reported that in some instances, tumor cells may remain dormant as solitary cells.^{145, 146} [↑ TOP](#)

Molecular components of the angiogenic switch

The first proteins that regulate angiogenesis were discovered in the early 1980s.

Promoters of angiogenesis

The observation in the 1970s that tumors implanted into the avascular cornea or onto the vascularized chick chorioallantoic membrane induced an ingrowth of new capillaries indicated that tumors released diffusible angiogenic factors.⁴⁶ This result motivated the development of in vitro and in vivo bioassays to guide the search for tumor-derived angiogenic factors.¹⁴⁷⁻¹⁴⁹ [↑ TOP](#)

Fibroblast growth factors

Basic fibroblast growth factor (bFGF or FGF-2) was the first angiogenic protein to be isolated and purified from a tumor (1982), followed shortly by acidic FGF (aFGF or FGF-1).^{29,150-152} There is extensive literature on the FGFs and their receptors (reviewed in Amalric and colleagues).¹⁵³ Acidic and bFGFs stimulate endothelial cell mitosis and migration in vitro and are among the most potent angiogenic proteins in vivo. They have high affinity for heparin and heparan sulfate, are stored in extracellular matrix, but lack a signal sequence for secretion.¹⁵⁴ The expression of bFGF receptors is very low. Although many different cells synthesize bFGF, including tumor cells of the central nervous system, sarcomas, genitourinary tumors, and even endothelial cells in the tumor vasculature,

it is not clear how bFGFs, in the absence of a signal sequence, are exported from tumors, unless proteinases or heparanases mediate release of FGF from extracellular matrix. ^{143,154-158} Recent work on identification of an FGF-binding protein secreted by tumors into the extracellular matrix may illuminate a mechanism of tumor mobilization of stored FGFs. ^{159, 160, 161} Furthermore, some tumors recruit macrophages ¹⁴⁰ and activate them to secrete bFGF, ¹⁴³ whereas others attract mast cells, which, because of their high content of heparin, could sequester bFGF. In spontaneous tumors that arise in transgenic mice, aFGF and bFGF are exported into conditioned medium by angiogenic tumor cells but not by pre-angiogenic cells in earlier stages of tumor progression. ^{54, 120} bFGF is not a specific endothelial mitogen, but has several cell targets including fibroblasts, smooth-muscle cells, and neurons. Therefore, it is puzzling why experimental tumors transfected with bFGF containing an engineered signal sequence stimulate mainly endothelial proliferation almost to the exclusion of smooth muscle and fibroblast proliferation. ^{61, 163} However, the ability of endothelial-derived angiopoietin-2 to repel smooth muscle or to prevent smooth muscle or pericytes from intimate contact with endothelial cells may explain this phenomenon in part (see Angiopoietins below). ¹⁶⁴⁻¹⁶⁶

bFGF interferes with adhesion of leukocytes to endothelium, and it has been suggested that tumors that elaborate bFGF may produce a form of local immunologic tolerance. ¹⁶⁷⁻¹⁶⁹

Abnormally elevated levels of bFGF are found in the serum and urine of cancer patients and in the cerebrospinal fluid of patients with different types of brain tumors. ^{170, 171} High bFGF levels in renal carcinoma correlate with a poor outcome. ¹⁷² Also, bFGF levels in the urine of children with Wilms' tumor correlate with stage of disease and tumor grade. ¹⁷³ [↑ TOP](#)

VEGF/VPF

Dvorak first proposed that tumor angiogenesis is associated with increased microvascular permeability. ¹⁷⁴ This led to the identification of vascular permeability factor (VPF). ¹⁷⁵⁻¹⁷⁷ VPF was subsequently sequenced by Ferrara and shown to be a specific inducer of angiogenesis called vascular endothelial growth factor (VEGF). ¹⁷⁶⁻¹⁷⁹ Since then, more than 15 angiogenic inducers have been identified, most of them as tumor products (Table 11-2). ¹⁸⁰

VEGF is an endothelial cell mitogen and motogen that is angiogenic in vivo. ¹⁸¹⁻¹⁸³ Its expression correlates with blood vessel growth during embryogenesis and is essential for development of the embryonic vascular system. ¹⁸⁴⁻¹⁸⁶ VEGF expression also correlates with angiogenesis in the female reproductive tract, and in tumors. ¹⁸⁷⁻¹⁹⁰ VEGF is a 40 to 45 kDa homodimeric protein with a signal sequence secreted by a wide variety of cells and the majority of tumor cells. VEGF exists as five different isoforms of 121, 145, 165, 189, and 206 amino acids, of which (VEGF₁₆₅

is the predominant molecular species produced by a variety of normal and neoplastic cells). Two receptors for VEGF are found mainly on vascular endothelial cells, the 180 kDa fms-like tyrosine kinase (Flt-1) ¹⁹¹ and the 200 kDa human kinase insert domain-containing receptor (KDR) and its mouse homolog, Flk-1. ¹⁹² VEGF binds to both receptors, but KDR/Flk-1 transducers the signals for endothelial proliferation and chemotaxis. ^{7,193-196} Other structural homologues of the VEGF family have recently been identified, including VEGF-B, VEGF-C, VEGF-D, and VEGF-E. ^{197, 198} VEGF-C binds to Flt-4, which is preferentially expressed on lymphatic endothelium. ^{199, 200} Neuropilin-1, a neuronal guidance molecule, is a recently discovered receptor for VEGF₁₆₅, but not for VEGF₁₂₁. ²⁰¹ Neuropilin is not a tyrosine kinase receptor and is expressed on nonendothelial cells, including tumor cells. This allows VEGF that is synthesized by tumor cells to bind to their surface. Surface-bound VEGF could make endothelial cells chemotactic to tumor cells, or it could act in a juxtacrine manner to mediate cooption of microvessels by tumor cells. Neuropilin also binds placenta growth factor-2 (PlGF-2) and heparin is essential for the binding of VEGF₁₆₅ and PlGF-2 to neuropilin-1. ^{57, 202} The natural cell surface polysaccharide in vivo is heparan sulfate, not heparin. Heparan sulfate may act as a template to accelerate the interaction of VEGF or PlGF-2 with VEGF. VEGF expression by tumors is up regulated by hypoxia and is often elevated near areas of tumor necrosis. ²⁰³⁻²⁰⁷ Hypoxia activates a hypoxia inducible factor-1 (HIF-1)-binding sequence in the VEGF promoter, which leads to VEGF mRNA transcription and stability. ^{7, 205}

VEGF expression is also upregulated by the *ras* oncogene. The farnesyl transferase inhibitors inhibit *ras* expression. It has been suggested that at least one mechanism of their antitumor effect may be to inhibit angiogenesis by inhibiting VEGF expression. ²⁰⁸

VEGF expression is inhibited by the von Hippel Lindau (VHL) protein. ²⁰⁹ The VHL tumor suppressor gene is inactivated in patients with VHL disease and in most sporadic clear-cell renal carcinomas. ^{210, 211} The VHL gene negatively regulates a series of hypoxia-inducible genes, including the VEGF, platelet-derived growth factor (PDGF) B, and the glucose transporter GLUT1 genes. ^{209,212} When VHL is mutated or deleted, these genes are overexpressed even under normoxic conditions. A recent important therapeutic advance employed an inhibitor (SU5416) of the VEGF receptor (VEGFR2) to restore sight in a patient with von Hippel Lindau syndrome who had a hemangioblastoma in the retina of her only eye. ²¹³

VEGF also induces fenestrations in endothelium of small venules and capillaries and even in tissues where microvessels are not normally fenestrated. ^{212, 214} The increased permeability of tumor vessels as revealed, for example, by the edema of brain tumors, may be partly mediated by VEGF-induced fenestrations. ²¹⁵

It is possible that certain other positive regulators of angiogenesis may

operate through VEGF or be VEGF-dependent. It remains to be seen whether the high angiogenic activity of bFGF is somehow acting indirectly through another endothelial mitogen such as VEGF (see below), because of the following observations. First, bFGF induces the expression of VEGF.²¹⁶ Second, the two endothelial mitogens act synergistically^{216, 217, 218, 219} to stimulate capillary tube formation in vitro. Third, systemic administration of a soluble receptor for VEGF (Flk-1) partially blocks cornea angiogenesis induced by implanted bFGF (R. D'Amato and C. Kuo, unpublished data). Another positive regulator of angiogenesis, transforming growth factor (TGF)- β , may also be dependent on VEGF. VEGF mRNA and protein are induced in fibroblasts and epithelial cells by TGF- β .²²⁰ In contrast, mRNA for placental growth factor, an angiogenic protein related to VEGF, is not induced by TGF- β .

These recent findings suggest that the angiogenic effect of TGF- β is mediated partly by its induction of VEGF in tissues. A clinical implication of these studies is that angiogenesis inhibitors that block VEGF may inhibit other angiogenic promoters as well. [↑ top](#)

Angiopoietins

Angiopoietin-1 is a 70 kDa ligand that binds to a specific tyrosine kinase expressed only on endothelial cells, called Tie2 (also called Tek). A ligand for Tie1 has not been elucidated.²²¹⁻²²⁵ Like VEGF, angiopoietin-1 is an endothelial cell specific growth factor. Angiopoietin-1, however, is not a direct endothelial mitogen in vitro. Rather, it induces endothelial cells to recruit pericytes and smooth muscle cells to become incorporated in the vessel wall. Pericyte and smooth muscle recruitment are mediated by endothelial production of PDGF-BB (and probably other factors) when Tie2 is activated by angiopoietin-1.²²⁶ There is increased vascularization in mice that overexpress angiopoietin-1 in the skin.²²⁴ The vessels are significantly larger than normal and the skin is reddened. The vessels are not leaky and there is no skin edema, in contrast to dermal vessels of mice overexpressing VEGF. In double transgenic mice expressing both angiopoietin-1 and VEGF in the skin, dermal angiogenesis is increased in an additive manner, but the vessels do not leak.²²⁷ This model closely approximates angiogenesis in healing wounds (ie, relatively nonleaky vessels with pericytes and some perivascular smooth-muscle cells contained in the vascular wall). In contrast, tumor vessels are leaky and thin-walled with a paucity of pericytes. Angiopoietin-2, produced by vascular endothelium in a tumor bed, blocks the Tie-2 receptor and acts to repel pericytes and smooth muscle.²²⁵ Nevertheless, tumor vessels remain thin "endothelium-lined tubes" even though some of these microvessels reach the diameter of venules (Figure 11-4). A key point is that angiopoietin-2 and VEGF together increase angiogenesis. However, if VEGF is antagonized or withdrawn at this point, endothelial cells may undergo apoptosis and new microvessels can regress.²²⁷

It is useful for clinicians to think of two classes of angiogenesis inhibitors: direct and indirect (Figure 11-5). [37](#) [↑ top](#)

Indirect angiogenesis inhibitors

These inhibitors generally decrease or block expression of a tumor cell product, neutralize the tumor product itself, or block its receptor on endothelial cells ([Figure 11-6](#)). Many of these tumor cell proteins are the products of oncogenes that drive the angiogenic switch. Rak and Kerbel reviewed the impact of 15 different oncogenes on tumor angiogenesis ([Table 11-3](#)).^{37,228–230} In vitro assays were originally employed to elucidate the activities of oncogene and tumor suppressor gene products, including, proliferation of cancer cells, resistance to apoptosis, immortalization, and anchorage independence.^{228, 229} In these in vitro assays, increased cancer cell proliferation and decreased apoptosis associated with oncogene mutations correlated so well with tumor growth in vivo, there was at first no indication that oncogenes could also upregulate angiogenic activity in tumor cells.²³¹ Nor was there any basis to predict that novel anticancer drugs, which inhibit oncogene activity by inhibition of its signal transduction, (eg, signal transduction inhibitors such as Herceptin) could also block proangiogenic products of tumor cells.²³¹ Only recently has it been recognized that many oncogenes are proangiogenic, and that this may be a major component of their tumorigenic activity. The proangiogenic oncogenes (in addition to bcl-2) listed in [Table 11-3](#) upregulate expression by tumor cells of angiogenic proteins and/or downregulate inhibitors of angiogenesis.^{228, 232}

Other experimental studies in which transfection with oncogenes increase tumor cell output of proangiogenic proteins and/or decrease expression of negative regulators of angiogenesis, support this concept.^{76, 77, 99, 230} For example, when the bcl-2 oncogene was transfected into prostate cancer cells that were already capable of developing large neovascularized tumors, VEGF expression and microvessel density both increased significantly.²³² Therefore, drugs that target oncogenes, or their products, or the receptors of those products, not only inhibit proliferation of tumor cells and increase their susceptibility to apoptosis, but, most importantly, these drugs disrupt production of tumor angiogenesis. Even re-activation of the tumor suppressor p53, can inhibit angiogenesis by at least four different mechanisms discussed below.^{75, 161, 233, 234} It is not surprising then that certain anticancer drugs developed for their capacity to block an oncogene product (for example, inhibitors of the EGF receptor tyrosine kinase), may have significant antiangiogenic activity, and that it will be "indirect" ([Tables 11-3](#) and [11-5](#)).^{228, 231, 235} For example, ras farnesyl transferase inhibitors block oncogene signaling pathways which upregulate tumor cell production of VEGF and downregulate production of the angiogenesis inhibitor, thrombospondin-1.²⁰⁸

Why emphasize in this chapter that anticancer drugs that target an oncogene product may inhibit tumor angiogenesis? Because a clinician armed with this knowledge should be better able to optimize the dose and schedule of an "antioncogene drug" or a "signal transduction inhibitor" by following antiangiogenic guidelines.³⁷ For example, if mutant tumor cells that express an angiogenic protein different from the one blocked by an "indirect" angiogenesis inhibitor arise, the indirect angiogenesis inhibitor may be discontinued prematurely, on the assumption that the tumor had

become drug resistant, and because conventional chemotherapy is traditionally discontinued in the presence of acquired drug resistance (see [Figure 11-6](#)). However, it may be prudent to continue a drug with significant antiangiogenic activity, especially if it is not toxic, and add a second angiogenesis inhibitor. For example, trastuzumab (Herceptin), an antibody that blocks HER-2/neu receptor tyrosine kinase signaling, suppresses cancer cell production of angiogenic factors such as TGF- β , angiopoietin-1, and plasminogen activator inhibitor-1 (PAI-1) and possibly VEGF. ^{231, 235} Herceptin also up-regulates expression of an endogenous angiogenesis inhibitor, thrombospondin, which may be the important basis of its antiangiogenic activity. ²³⁵ If tumor cells begin to express a different angiogenic protein such as bFGF, or interleukin-8 the tumor under treatment may appear to have become "resistant" to Herceptin. ^{236, 237} A similar speculation may apply to certain epidermal growth factor (EGF) receptor inhibitors such as Iressa (gefitinib; ZD 1839), or to other tumor cell products which regulate angiogenesis (see [Table 11-3](#)). ^{237, 238}

Of interest is that certain tumors such as giant cell bone tumors and angioblastomas, generate only or mainly bFGF, without usually expressing other angiogenic proteins. When patients with these tumors were treated with interferon- α at low daily doses, drug resistance was not observed with therapy of 1 to 3.5 years duration. ²³⁹⁻²⁴² Interferon- α at low doses inhibits bFGF production by tumor cells and would therefore be considered an indirect angiogenesis inhibitor. ²⁴³ However, interferon also inhibits endothelial cell motility directly, and therefore can be considered to have both direct and indirect antiangiogenic activity. ²⁴⁴ [↑ TOP](#)

Direct angiogenesis inhibitors

These inhibitors directly block vascular endothelial cells from proliferating, migrating or increasing their survival in response to a spectrum of proangiogenic proteins, including VEGF, bFGF, IL-8, PDGF, and PD-ECGF and others ([Table 11-4](#)). Direct angiogenesis inhibitors are less likely to induce acquired drug resistance, because they target genetically stable endothelial cells, rather than unstable mutating tumor cells. ²⁴⁵ Tumors in mice treated with antiangiogenic therapy did not develop drug resistance. ⁶⁹ This does not mean that angiogenesis inhibitors are less susceptible to metabolic degradation, increased clearance from the blood stream, or development of neutralizing antibodies, than any other drug that is administered systemically.

Direct angiogenesis inhibitors include (1) certain synthetic inhibitors or peptides designed to interfere with various steps in the angiogenic process (eg, antagonists of the integrin $\alpha_v\beta_3$); or inhibitors of metalloproteinases; (2) low molecular weight compounds (eg, TNP-470), thalidomide, angiostatic steroids such as tetrahydrocortisol, 2-methoxyestradiol, and squalamine; and (3) endogenous (natural) proteins, which generally prevent vascular endothelial cells from responding to a wide spectrum of angiogenic promoters (eg, interferon- α , interleukin-12, platelet factor 4, thrombospondin-1, angiostatin, endostatin, arresten, canstatin, tumstatin,

PEX, pigment epithelium-derived factor, and antiangiogenic antithrombin III (an internal fragment of antithrombin III, named aaAT). [28](#), [59](#), [64](#), [65](#), [67](#), [70](#), [246](#), [247–266](#) [↑ top](#)

Endogenous Inhibitors of Angiogenesis

Angiostatin, endostatin, tumstatin, arresten, canstatin and aaAT are specific angiogenesis inhibitors that are endogenous. The rationale that led to their discovery is discussed here in detail because (1) these proteins have profoundly changed our thinking about how primary tumors and metastases regulate their own growth; (2) the method of their discovery provides a unique strategy that is leading to the elucidation of an enlarging family of endothelial inhibitor proteins which, under physiologic conditions, may act to limit or prevent angiogenesis; and (3) endostatin and angiostatin are currently in Phase II clinical trials.^{[267](#)} Tumstatin is a candidate for future clinical trials (see Clinical Trials below).

The first clue to the existence of endogenous angiogenesis inhibitors came from the discovery that interferon- α - β inhibited endothelial cell migration and that platelet factor-4 inhibited endothelial proliferation. [244](#), [260](#), [268](#) Both were subsequently shown to inhibit angiogenesis. [244](#), [260](#), [268–270](#)

However, Rastinejad and colleagues were the first to show that a tumor could generate an angiogenesis inhibitor. They subsequently proposed that the angiogenic phenotype was the result of a net balance of endogenous inhibitors and stimulators of angiogenesis. ^{[271](#)} A nontumorigenic hamster cell line became tumorigenic in association with the loss of a suppressor gene and concomitant with the onset of angiogenic activity. The nontumorigenic line secreted high levels of an angiogenesis inhibitor, a truncated form of thrombospondin-1 (TSP-1), that decreased by about 96% in the tumorigenic cells. ^{[261](#)} TSP-1 was shown to be regulated by the wild-type tumor suppressor p53 in fibroblasts and in mammary epithelial cells. ^{[75](#), [272](#)} Loss of p53 function in the transformed derivatives of these cells dramatically decreased the level of angiogenesis inhibitor. Restoration of p53 upregulated TSP-1 and raised the antiangiogenic activity of the tumor cells. Deletion of thrombospondin-1 leads to accelerated growth of breast cancers that arise spontaneously in neurotransgenic mice. ^{[273](#)} The demonstration by Rastinejad and coworkers that the switch to an angiogenic phenotype involved a negative regulator of angiogenesis generated by the tumor per se suggested to Folkman a unifying angiogenic mechanism to explain a well-recognized but previously unsolved clinical and experimental phenomenon: the inhibition of tumor growth by tumor mass. A description of this phenomenon is quoted from the introduction of Folkman's paper. ^{[65](#)} "The removal of certain tumors, eg, breast carcinomas, colon carcinomas, and osteogenic sarcomas can be followed by rapid growth of distant metastases."^{[274–277](#)} Postoperative chemotherapy was introduced mainly to prevent or delay the growth of secondary metastases. Several studies in terminally ill patients demonstrate the suppression of a secondary tumor by a primary tumor.^{[278](#)} ^{[279](#)} A primary tumor can suppress metastases originating from a different type of tumor (eg, a breast cancer can inhibit melanoma metastases). In

melanoma, partial spontaneous regression of the primary tumor may be followed by rapid growth of metastases. When ionizing radiation is employed to regress a small-cell lung cancer, distant metastases may undergo rapid growth.^{70, 280} If one portion of a primary tumor is removed (eg, cytoreductive surgery for testicular cancer), the residual tumor increases its rate of expansion.²⁸¹ The same phenomenon is observed in several different animal tumors, that is, that some primary tumors may inhibit the growth but not the number of their metastases.²⁸²⁻²⁸⁶ Partial removal of a primary tumor increases growth rate of the residual tumors similar to humans.^{287, 288} Furthermore, metastatic growth can suppress the growth of a primary tumor (analogous to the occult primary in a cancer patient).²⁸⁹ Many primary tumors can suppress the growth of a second tumor inoculation.²⁹⁰⁻²⁹⁷ This "resistance" to a second tumor challenge is inversely proportional to the size of the tumor inoculum and directly proportional to the size of the first tumor. A threshold size is necessary for the inhibitory effect to occur, and some primary tumors can inhibit a secondary tumor of a different type.^{295, 297}

At least three hypotheses have been advanced to explain these diverse observations and experiments: (1) "concomitant immunity," in which a primary tumor induces an immunologic response against a secondary tumor or a metastasis in the same host, (2) depletion of nutrients by the primary tumor, or (3) production of antimitotic factors from the primary tumor that directly inhibit the proliferation of the secondary tumor.^{291, 295, 298-300} None of these concepts, however, offered a molecular mechanism to explain all of the experiments cited above, in which tumor growth is suppressed by tumor mass. Once it was demonstrated that a tumor could generate a negative regulator of angiogenesis, then it became clear that a primary tumor, while stimulating angiogenesis in its own vascular bed, could possibly inhibit angiogenesis in the vascular bed of a distant metastasis.²⁶¹ However, at least two conditions would be necessary: first, the primary tumor (ie, the first tumor to grow) would need to generate an angiogenic promoter in excess of an inhibitor in its own vascular bed and, second, the putative inhibitor would need to have a longer half-life in the circulation than the angiogenic promoter. After arriving in my laboratory in the summer of 1991, Michael O'Reilly validated this hypothesis by discovering angiostatin, endostatin, and antiangiogenic antithrombin over the next 8 years.^{65-67, 69, 70, 301}

Angiostatin

Angiostatin is a 38 kDa internal fragment of plasminogen that was purified from serum and urine of mice bearing a subcutaneous Lewis lung carcinoma that suppressed growth of its lung metastases by inhibiting their angiogenesis (Figure 11-7).⁶⁵ Lung metastases remained microscopic and did not grow beyond approximately 200 μ m in diameter. They were not neovascularized and usually formed a microcylinder around a single microvessel. In these dormant metastases, 38% of the tumor cells were proliferating (DNA synthesis was determined by bromodeoxyuridine [BrdU] labeling) and 7 to 8% were undergoing apoptosis.⁴⁵ The presence of a circulating angiogenesis inhibitor was evidenced by the almost

complete inhibition of bFGF-stimulated corneal neovascularization in these mice. Further, serum from these tumor-bearing mice specifically inhibited capillary endothelial cell proliferation *in vitro* by more than 70% (but not fibroblast, smooth-muscle cell, epithelial cell or tumor cell proliferation). The tumor-bearing serum also inhibited angiogenesis on the chick chorioallantoic membrane compared to serum from mice without tumors (which stimulated endothelial cells). After removal of the primary tumor, serum endothelial inhibitory activity disappeared by 6 days (half-maximal 2.5 days), lung metastases were neovascularized by 5 days, and by 15 days the mice were dying of large vascularized metastases. There was no change in tumor cell proliferation (approximately 38% BrdU-labeled cells), but tumor cell apoptosis fell to 2%. Systemic administration of angiostatin purified from mouse urine significantly inhibited angiogenesis in lung metastases and restricted their growth to a microscopic dormant size,⁶⁵ and recombinant angiostatin potently inhibited growth of other tumor types.⁶⁶ Again, tumor cell proliferation remained as high as in the tumor-bearing mice, and apoptosis was significantly reduced.^{45, 48} Angiogenesis inhibitor in the urine of tumor-bearing mice was completely removed by angiostatin neutralizing antibodies. A subline of Lewis lung carcinoma incapable of inhibiting metastatic growth did not generate angiostatin or inhibit corneal angiogenesis, nor did its serum inhibit endothelial cell growth. VEGF, the major angiogenic promoter in Lewis lung carcinoma, has a half-life of approximately 3 minutes in the circulation.

Angiostatin is not secreted by tumor cells but is generated through proteolytic cleavage of circulating plasminogen by a series of enzymes released from the tumor cells. At least one of these tumor-derived enzymes, urokinase plasminogen activator (uPA), converts plasminogen to plasmin, while a phosphoglycerate kinase from hypoxic tumor cells then reduces the plasmin so that it can be converted to angiostatin by one of several different metalloproteinases (Figure 11-8).³⁰² Other types of tumors have since been reported to generate angiostatin (eg, human prostate cancer).³⁰³⁻³⁰⁵ Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor angiostatin.³⁰² Prostate-specific antigen generates angiostatin-like fragments from plasminogen.³⁰⁷

Transfection of Angiostatin.

Furthermore, when murine fibrosarcoma cells were transfected with angiostatin, primary subcutaneous tumors formed whose growth was slowed in proportion to increased levels of angiostatin production by the tumor cells. In these tumors, the total angiogenic output of the primary tumor was decreased by transfected angiostatin, which opposed in a dose-dependent manner the activity of the tumor's secreted angiogenic promoter, but never completely counteracted it.⁷⁸ It should be emphasized that the rate of tumor growth (expansion of tumor mass) was directly proportional to total angiogenic output of the tumor, inversely proportional to angiostatin production and to tumor cell apoptosis, and virtually independent of tumor cell proliferation. This result was confirmed in a different tumor model, Kaposi's sarcoma subjected to

angiostatin gene transfer, which resulted in sustained angiostatin expression, delayed tumor growth and reduced tumor vascularization.³⁶⁰ Gene transfer with a different angiogenesis inhibitor, thrombospondin, gave similar results. Streit and colleagues reported (in an experiment discussed above) that when tumor cells were transfected with the angiogenesis inhibitors thrombospondin-1 and/or thrombospondin-2, proliferation rate (as determined by staining for the proliferating cell nuclear antigen [PCNA]) (see [Figure 11-9](#)), also remained relatively constant, despite the decreased angiogenesis and suppressed tumor growth.⁷⁹ [↑ top](#)

Mechanisms of Action.

Angiostatin and its isoforms induce cell arrest and apoptosis of endothelial cells and inhibit endothelial migration, angiogenesis in vitro, and angiogenesis in the quail chorioallantoic membrane, which provides a quantitative bioassay.³⁰⁸⁻³¹⁷ They can also be generated by different enzymes and by other cell types; can inhibit other tumor types; decrease activity of the mitogen-activated protein kinase ERK-1 and ERK-2 in endothelial cells; up-regulate E-selectin in proliferating endothelial cells; and can be delivered in vivo by gene therapy.^{302-304,313-333} In addition, they bind specifically to ATP-synthase, a transmembrane protein expressed by vascular endothelial cells; bind to a fragment of vitronectin; and can potentiate radiation therapy of experimental tumors.³³⁴⁻³³⁷

A provocative recent finding is that proliferation of circulating precursor endothelial cells derived from bone marrow is inhibited at significantly lower concentrations of angiostatin than is proliferation of endothelial cells isolated from tissues.¹³⁶ Because precursor endothelial cells from bone marrow can home to angiogenic sites and participate in new vessel formation in a tumor (see above), it has been speculated that at least one mechanism of angiostatin is to inhibit this subpopulation of endothelium, and that these cells may be employed as a sensitive bioassay for identification of novel antiangiogenic molecules.¹³⁶ Angiostatin effectively blocks endothelial cell migration induced by plasmin binding to $\alpha_v\beta_3$ integrin.^{346, 362} Angiostatin may act at least in part by transiently increasing ceramide, a sphingolipid second messenger implicated in a proapoptotic pathway, and by increasing RhoA, an effector of cytoskeletal structure.³⁶¹

Numerous additional recent studies have been reported on the mechanism of angiostatin and on its biological and pharmacological activities. A few of these new findings are discussed here. Angiostatin, but not plasminogen, specifically binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells.³³⁸ This affinity appears to be specific for endothelial cells and does not occur with fibroblasts. Annexin II on endothelial cells organizes assembly of plasminogen on its surface and produces plasmin at maximum efficiency in the vascular bed.³³⁸⁻³⁴¹ Plasmin is a serine protease that degrades fibrin and several endothelial cell matrix proteins, such as laminin, thrombospondin, and collagens, and facilitates capillary sprouting during

angiogenesis as well as endothelial migration.^{338, 346, 362} In fact, angiostatin has been shown to inhibit endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation (see [Figure 11-8](#)).³²⁶ However, because hypoxic tumor cells produce a phosphoglycerate kinase that enzymatically reduces plasmin, this mechanism contributes to the generation of angiostatin, which can then block plasmin organization on the endothelial surface and interfere with angiogenesis.^{302, 363} The gene for annexin II is also upregulated in endothelial cells during angiogenesis. Annexin II is a central molecule in signal transduction mechanisms, and disruption of its function affects calcium signaling, tyrosine phosphorylation, cell proliferation and apoptosis.³⁴²⁻³⁴⁴ Angiostatin mediation of endothelial cell death appears to be through a calcium signaling mechanism.³⁴⁵ Therefore, annexin II may act as an endogenous angiogenic switch, and its blockade may be at least one potential mechanism for angiostatin. [↑ top](#)

Binding proteins for angiostatin.

Three other binding proteins have been suggested as putative receptors for angiostatin: (1) ATP synthase on the endothelial surface, (2) aVb3, and (3) angiotonin, which belong to a novel protein family with conserved coiled-coil and PDZ binding domains.^{334,346-348} Taken together with annexin II, Tuszynski and colleagues suggest that angiostatin may bind to multiple receptors and could mediate a cascade of events leading to inhibition of angiogenesis.³³⁸ Angiostatin also inhibits signaling of hepatocyte growth factor (HGF) in endothelial cells (and in smooth muscle cells), by blocking HGF-induced signaling of c-met, Akt, and ERK1/2.³⁴⁹ However, angiostatin does not block VEGF or bFGF signaling events in endothelial cells. Angiostatin also induces intracellular acidosis in endothelial cells and anoikis (loss of cell attachment) in endothelial cells that are incubated under conditions resembling the low pH in a tumor.³⁵⁰ Angiostatin downregulates expression of VEGF expression in tumor cells.³⁵¹ This implies that angiostatin may act not only as a direct inhibitor of angiogenesis, but also as an indirect angiogenesis inhibitor. Analysis of the x-ray crystallographic structure of angiostatin reveals that kringles 1, 2, and 3 produce a central cavity suggesting a unique domain where they may function in concert.^{352, 364} The enzymatic mobilization of angiostatin is facilitated by tumor-derived matrix-metalloproteinase.³⁵³ Other labs confirm the report that circulating angiostatin generated by a primary tumor decreases significantly after the primary tumor is removed and that this is followed by growth of remote metastases.^{35, 65} [↑ top](#)

Angiostatin gene therapy.

Angiostatin gene therapy shows efficacy in tumor-bearing mice against a variety of tumors and their metastases including, breast cancer, renal cell cancer, gliomas, melanoma, Kaposi's sarcoma, squamous cell carcinoma, and leukemia.^{310, 360,365-375} Of interest was that viral vector-mediated antiangiogenic therapy using an angiostatin-endostatin fusion protein demonstrated significant downregulation of ascites, tumor growth, vascularity and prolongation of animal survival in three different ascites

tumor models.³⁷⁶ Angiostatin has been transfected into canine eyes as a preclinical model for the treatment of human uveal melanoma.³⁷⁷ Long-term expression of angiostatin and endostatin from lentivirus-transduced human bladder carcinoma cells inhibited proliferation of endothelial cells co-cultured with the tumor cells, the first use of a lentiviral vector for antiangiogenic gene delivery.³⁷⁸ Angiostatin gene therapy has also shown efficacy in nonneoplastic mouse models of disease such as arthritis, retinal neovascularization, choroidal neovascularization, and endometriosis.³⁷⁹
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Angiostatin protein therapy.

Angiostatin protein therapy has shown significant efficacy when delivered systemically to mice bearing a variety of different tumors, and also in combination with endostatin where it has shown a synergistic effect or as a fusion protein of angiostatin and endostatin, or in combination with interleukin-12.^{351,383-387} When three different types of murine tumors were engineered to produce granulocyte colony-stimulating factor, (GM-CSF), the level of angiostatin in the serum increased 4-fold above controls and directly correlated with GM-CSF production.³⁸⁸ Angiostatin levels directly correlated with macrophage metalloelastase production, which appeared to mediate cleavage of angiostatin from plasminogen. Metastases were suppressed in all three tumor systems. [↑ TOP](#)

Replacement of angiostatin after radiotherapy.

Radiotherapy of primary Lewis lung carcinomas in mice induced regression of the primary tumor, but led to disappearance of angiostatin from the blood and rapid growth of lung metastases.²⁸⁰ Angiostatin replacement therapy prevented growth of lung metastases after regression of the primary tumor by radiotherapy. In some patients successful radiotherapy of a primary tumor is followed by growth of distant metastases, for example, nonsmall cell lung cancer. It may eventually be possible to determine if a tumor is generating an endogenous angiogenesis inhibitor prior to radiotherapy and then administer that inhibitor in combination with the radiotherapy.

Angiostatin protein also inhibits angiogenesis in nonneoplastic states. For example, angiostatin inhibits corneal neovascularization, corpus luteum development in the pre-ovulatory follicle, and as pathological retinal angiogenesis, but not physiological retinal neovascularization.³⁸⁹⁻³⁹² Although the original report of 38 kDa angiostatin included kringles 1-4 of plasminogen, a recent report reveals the generation of a novel plasminogen fragment of 22 kDa generated by enzymatic activity from tumor cells.^{65, 393} The p22 plasminogen fragment includes kringle 1 plus additional N- and C- terminal residues, but circular dichroism and intrinsic fluorescence analysis define structural differences between it and recombinant kringle 1. Angiogenesis and tumor growth are potently inhibited by p22.

When a strategy similar to the one that uncovered angiostatin (eg, suppression of a tumor growth by tumor mass) was employed by O'Reilly

and colleagues with murine hemangioendothelioma and human small-cell lung cancer, endostatin and antiangiogenic antithrombin (aaAT) were discovered. ^{67, 69, 70} Both endostatin and aaAT are generated from larger parent proteins by enzymes released by the tumor cells. Angiostatin is currently in phase I/II clinical trials. [↑ top](#)

Endostatin

Endostatin is a 20–22 kDa internal fragment of collagen XVIII. ^{67, 69, 394, 395} It is the first of a group of endogenous angiogenesis inhibitors which are predominately extracellular proteins, which generally require proteolytic processing to become active. ^{396, 397} More than 350 reports on endostatin have been published in the 5 years since its isolation and identification as an angiogenesis inhibitor. ⁶⁷ These range from mechanistic studies to experimental therapeutics. A variety of new findings have been reported on mechanism of action, physiologic function, structure-activity relationships, experimental therapeutics including gene therapy, and early clinical studies. A few of these studies are reviewed here.

Structure-activity relationships.

Endostatin was first isolated and sequenced from conditioned medium of murine hemangioendothelioma based on the same strategy employed for the discovery of angiostatin (ie, hemangioendothelioma suppressed secondary tumors). ³⁹⁸ It is a specific inhibitor of endothelial cell proliferation and migration like angiostatin. High-resolution radiographic structures have been determined for mouse and for human endostatin (Figure 11-10). ^{399, 400} At least two enzymes produced by tumor cells are necessary to cleave endostatin from collagen XVIII, an elastase and a cathepsin. ^{401, 402} Endostatin is derived from the nontriple helical C-terminal NCI domain of collagen XVIII. Subsequently collagen XV has also been found to share a C-terminal NC1 domain containing the endostatin molecule, which has not been found to date in any other proteins. ⁴⁰³ Endostatin is released proteolytically in trimeric form and further converted to monomeric forms of about 20 kDa. Both endostatin isoforms share a compact globular fold, but differ in binding properties for proteins and cells. ⁴⁰³ Tissue distributions are also different. Differences in activity have been found between NCI domains and endostatin activity may be related to its oligomerization state. [↑ top](#)

Endostatin in basement membrane.

Endostatin is present in basement membranes and vessel walls and is especially rich in elastic fibers of the aorta and sparse elastic fibers of veins. ⁴⁰⁴ Some, but not all, capillaries or arterioles show weak labeling for endostatin. Within the elastic fibers, endostatin is co-localized and has been reported to bind to fibulin-1 and -2, nidogen-1 and -2, laminin-1 and perlecan. ⁴⁰⁵ Recombinant mouse endostatin binds to heparin with a K_d of 0.3 μ M. ⁴⁰⁶ A major site of four clustered arginines, 155, 158, 184 and 270 and a second site (R193, R 194) is essential for binding to heparin as

well as to heparan sulfate and sulfatides, but not to fibulin-1 and fibulin-2. A minimum heparin size of 12-mer (dodecasaccharide) is necessary for efficient binding and there is a crucial role for 2-O and 6-O sulfation. A synthetic arginine-rich dendrimer that mimicked the surface of endostatin and had high affinity for heparin, had similar antiangiogenic activity in the chick embryo. This experiment demonstrates the important role of heparin affinity for the inhibitory activity of endostatin.⁴⁰⁷ A decreased inhibitory activity after mutation of the zinc-binding site was reported for insoluble recombinant endostatin from *E. coli*,⁴⁰⁸ but elimination of zinc binding had no effect on antiangiogenic activity in mouse soluble recombinant endostatin.⁴⁰⁶ Also, zinc ligand-disrupted recombinant soluble human endostatin from yeast showed potent antitumor activity in mice.⁴⁰⁹ It is unclear why insoluble recombinant endostatin required zinc for inhibitory activity,⁴⁰⁸ but that soluble endostatin did not. However, it should not be overlooked, that the original insoluble recombinant zinc-containing endostatin induced more rapid tumor regression in different tumor types than has been obtained to date with soluble forms with or without zinc.^{67, 69} One possibility is that zinc is required to maintain folding of the insoluble protein.⁴⁰³ Alternatively we have preliminary data that peptides of endostatin released from insoluble endostatin inhibit angiogenesis (K. Javaherian, personal communication). Furthermore, under physiological conditions endostatin exists mainly as a fragment of an insoluble matrix protein, collagen XVIII. It appears that only a small fraction of endostatin circulates in the soluble form (ie, in the range of 30 ng/mL).

Of the 20 collagen isoforms that have been identified so far in mammalian species, collagens IV, XV and XVIII (which possess C-terminal globular domains) have been implicated in regulation of angiogenesis.⁴¹⁰ Of interest is that homologues of these three collagens have also been identified in the worm *C. elegans*, where the endostatin domain of type XVIII collagen controls migration of neuronal cells and axon guidance instead of vascular endothelial cells.⁴¹¹ The evolutionary age of endostatin of approximately 600 million years, may be responsible in part, for its lack of toxicity in animals and patients.^{67, 69, 267} It is also fascinating from an evolutionary perspective that certain regulators of endothelial cell migration have (or had) control of axon migration, eg, endostatin, neuropilin semaphorins and ephrins, among others.⁴¹²⁻⁴¹⁵ This relationship provides insight into how development of nerves and blood vessels may be coordinated.

In the chick embryo angiogenesis induced by bFGF was inhibited by endostatin, but not by an endostatin mutant that does not bind to heparin. [↑ TOP](#)

Mechanisms of action.

Novel mechanisms have recently been reported for the antiangiogenic action of endostatin, although we are a long way from a complete picture.

Inhibition of VEGF receptor.

Endostatin blocks the binding of VEGF₁₂₁ and VEGF₁₆₅ to the KDR/Flk-1 receptor, blocks tyrosine phosphorylation of this receptor, and blocks activation of its intracellular signaling events, ERK, p38 MAPK and p125FAK.⁴¹⁶ This receptor mediates endothelial cell motility and proliferation. Endostatin also blocks VEGF. [↑ top](#)

Downregulation of VEGF expression.

While endostatin does not bind to VEGF,⁴¹⁶ it does downregulate VEGF expression in tumor cells (similar to the effect of angiostatin).³⁵¹ Endostatin can therefore be considered to act as both a 'direct' and an 'indirect' angiogenesis inhibitor. In bFGF-treated endothelial cells, endostatin induces endothelial cell apoptosis, in part by activating tyrosine kinase signaling of the Shb adaptor protein.⁴¹⁷ However, endostatin does not compete with binding of bFGF to tissues, and it does not affect bFGF receptor signaling.⁴¹⁸ This result is consistent with the observation that bFGF (FGF-2) stimulation of endothelial cell proliferation in vitro and angiogenesis in vivo is mediated by VEGF.⁴¹⁹ [↑ top](#)

Prevention of bFGF-induced or VEGF-induced loss of endothelial cell-cell adhesion or endothelial cell adhesion to basement membrane.

Endothelial cell migration, which is critical for new sprout formation during angiogenesis, requires continuous turnover of cell-cell interactions and of cell-matrix interactions.⁴²⁰ Cell-cell interactions between endothelial cells are maintained in part by β -catenin localized at cell-cell junctions in cadherin complexes of capillary endothelial cells in vitro. Treatment of these cells with bFGF shifts β -catenin to the cytoplasm, which loosens cell-cell adhesion and facilitates endothelial migration.⁴²¹ However, co-treatment with endostatin transiently blocks this shift.⁴²¹ Cell-matrix interactions are maintained in part by formation of focal adhesions and actin stress fibers. Either bFGF or endostatin alone, induced tyrosine phosphorylation of focal adhesion kinase and paxillin, which promoted formation of focal adhesions and actin stress fibers. However, when microvascular endothelial cells were co-treated with bFGF and endostatin, focal adhesion and actin stress fibers were decreased, which decreased migration of endothelial cells. In an in vivo situation, the entire vascular bed would be exposed to circulating endostatin. But its antiangiogenic effect would only become evident in areas of high growth factor stimulation, such as those present in the tumor endothelium.⁴²¹ Taken together, these results provide a plausible mechanism to explain clinical trial data (see below) in which antiangiogenic effects of endostatin are observed in the absence of systemic toxicity.⁴²¹ [↑ top](#)

Vessel stabilization: reversal of bFGF-induced or VEGF-induced loss of endothelial cell-cell adhesion or endothelial cell adhesion to basement membrane.

Another antiangiogenic mechanism of endostatin in addition to its antiproliferative and antimotility effect on angiogenic endothelial cells is the stabilization of newly formed endothelial tubes. Endostatin decreases

formation of VEGF-induced microvessels sprouting from aortic rings in vitro. By immunohistochemistry, administered endostatin localizes to endothelial cell-cell junctions as well as to adhesion sites between endothelial cells and their basement membrane, thus anchoring endothelial cells to each other and to their basement membrane (Figure 11-11).⁴²² Endostatin does not localize to quiescent vasculature. High concentrations of endostatin were detected by immunohistology in rat and human brain tumors in and around endothelial cells, in tumors cells, and in macrophages and leukocytes in the tumor's vascular bed, but normal brain showed no endostatin reactivity.⁴⁴⁷ This unique localization of endostatin only in newly formed angiogenic microvessels, appears to reverse the loosening of endothelial cells to each other and to their basement membrane, which was necessary for them to form angiogenic sprouts in the first place. Similar "stabilizing" effects of endostatin were observed in tumors in vivo, where administered endostatin was more abundant in association with angiogenic vessels undergoing maturation, mainly at the tumor marginal zone where angiogenesis was highly active. Endostatin immunostaining is present during vascular formation up to the stage exhibiting one layer of peri-endothelial cells, but disappears in further stabilized vessels that have more than one layer of these cells in their wall.⁴²² This suggests that the vascular stabilizing effect of endostatin occurs earlier than the integration of peri-endothelial cells into the vascular wall. A subsequent step of vascular stabilization by recruitment and integration of peri-endothelial cells (pericytes and smooth muscle cells) is mediated by angiopoietin-1, TGF- β , and PDGF.²²⁶ Of interest is that during endothelial tube formation induced by VEGF, endostatin plus VEGF-treated tubes became so stable that after cessation of endostatin, the survival time of these tubes was approximately doubled in comparison to VEGF alone. Furthermore, incubation of endothelial cells with an antibody to endostatin prevented formation of stable VEGF-induced tubes and disrupted existing tubes. The role of endostatin in modulating endothelial cell interaction with extracellular matrix is also revealed by experiments in which oligomeric endostatin (from the NC1 domain of collagen XVIII), induces motility and morphogenesis of tube forming endothelial cells in Matrigel, whereas monomeric endostatin inhibits these effects.⁴²³ Endostatin downregulates the uPA system on the endothelial cell surface.⁴²⁴ Pro-angiogenic proteins such as bFGF increase the secretion of uPA and plasminogen activator inhibitor (PAI-1) leading to an overall increase in proteolytic activity prerequisite for endothelial cell sprouting and tube formation. Endostatin treatment of endothelial cells markedly decreases their production of uPA and PAI-1. \uparrow TOP

Inhibition of metalloproteinase.

Endostatin also binds and inhibits the catalytic activity of MMP-2.^{425, 426} As a result, endothelial cell invasion is inhibited and tumor cell invasion may also be decreased. Tumor progression and angiogenesis are reduced in MMP-2 deficient mice and MMP-2 is required for the switch to the angiogenic phenotype.^{106, 427} \uparrow TOP

Binding of endostatin to endothelial cell surface integrins and other proteins that modulate motility and morphogenesis.

Endostatin also inhibits integrin-dependent endothelial cell migration because it binds to α_5 - and α_v -integrins on the endothelial cell surface, in particular $\alpha_5\beta_1$.⁴²⁸ Kalluri and colleagues have also proposed that $\alpha_5\beta_1$ -integrin may be a functional receptor for endostatin (R. Kalluri, unpublished data).³⁷ High concentrations of endostatin also interact with tropomyosin to interrupt microfilament integrity, which inhibits endothelial motility and may participate in part in endothelial apoptosis.⁴²⁹ Endostatin also binds to glypicans on the endothelial cell surface at concentrations which suggest that glypicans may be low affinity endostatin receptors.⁴³⁰ [↑ top](#)

Inhibition of cyclin D1 causing G1 arrest of endothelial cells.

Endostatin causes G1 arrest in endothelial cells, by decreasing the hyperphosphorylated retinoblastoma gene product and downregulating cyclin D1 mRNA and protein.⁴³¹ Transcription through the LEF1 site in the cyclin D1 promoter is essential for endostatin's inhibitory activity. Others report that endostatin arrests proliferation and causes apoptosis of endothelial cells.^{432, 433} [↑ top](#)

Dephosphorylation of endothelial nitric oxide.

Human recombinant endostatin dose-dependently blocked VEGF-induced endothelial cell migration, with complete inhibition at 100 ng/mL.⁴³⁴ VEGF stimulates synthesis of endothelial nitric oxide (eNOS). In endothelial cells sphingosine-1 phosphate activates the serine/threonine kinase Akt and stimulates phosphorylation of eNOS to release nitric oxide.⁴³⁵ VEGF-stimulated endothelial nitric oxide synthesis (eNOS) was abolished by endostatin, (although VEGF-induced formation of prostacyclin was not significantly affected by endostatin). Endostatin interfered with activation of eNOS downstream of Akt by specifically inhibiting phosphorylation of eNOS at Ser 1177 by stimulating activation of the phosphatase PP2A (Figure 11-12).⁴³⁴ Nitric oxide synthesis also appears to be critical for the mitogenic effect of VEGF on endothelial cells.^{114–116} Inhibition of nitric oxide synthesis also blocks tumor angiogenesis.⁴³⁶ We have previously proposed another role for nitric oxide synthesis in angiogenesis: early vasodilation of pre-existing quiescent vessels as a prerequisite for sprout formation.¹¹⁶ [↑ top](#)

Hypoxic downregulation of endostatin production by endothelial cells and pericytes.

Collagen XVIII is converted to endostatin, which is secreted from endothelial cells and pericytes in vitro at a constant rate.⁴³⁷ The rate of conversion is higher in pericytes. During hypoxia, production of endostatin is significantly decreased. (30–40% of this hypoxia-induced 43% degradation of endostatin occurs in pericytes and approximately 10% in endothelial cells.) Exogenous endostatin is more rapidly degraded when incubated with hypoxic conditioned media of endothelial cells or pericytes. The mechanism of endostatin degradation by the conditioned

medium is unknown. These results imply that: (1) decreased endostatin production by hypoxic pericytes and/or endothelial cells may be another mechanism to facilitate the angiogenic switch in a tumor; (2) pericytes may employ endostatin as one of the strategies to inhibit endothelial proliferation under normal conditions; and (3) the treatment of a patient with a large tumor burden or with a tumor with a large hypoxic center (eg, glioblastoma), may require high doses of endostatin at the beginning of therapy, because of endostatin degradation. [↑ top](#)

Physiologic Functions

Physiologic functions for endostatin are gradually being elucidated.

Regulation of development of retinal vasculature.

Endogenous endostatin in the eye may contribute in part to the normal development of vasculature in the retina. During development of the eye, hyaloid vessels supply the vitreous and the lens. Subsequently, these vessels regress leaving a transparent vitreous and lens. Endostatin is thought to play a key role in regression of the hyaloid vessels because they fail to regress in endostatin's absence. In a rare disease called Knobloch syndrome, the hyaloid vessels fail to regress, retinal vasculature fails to develop, and patients suffer retinal degeneration and blindness, among other abnormalities.^{438,439} In this syndrome, a splice mutation in human collagen XVIII leads to a truncated protein lacking the endostatin fragment. A proposed mechanism of retinal degeneration is that persistent hyaloid vessels increase oxygen in the retina, which suppresses hypoxia-inducible factor-1 (HIF-1 α) and VEGF, both of which drive normal vascular growth in the normal developing retina. Retinal vasculature also fails to develop in a collagen XVIII-deficient mouse model.⁴⁴⁰ Thus, a pathological deficiency of endostatin in humans and mice reveals that endostatin is an endogenous inhibitor of angiogenesis under normal physiological conditions. [↑ top](#)

Endostatin in platelets.

Platelets contain at least 14 positive regulators and approximately 12 negative regulators of angiogenesis.⁴⁴¹ The positive regulators include VEGF-A, VEGF-C, bFGF, HGF, angiopoietin-1, PDGF, heparanase and sphingosine-1 phosphate, among others. The negative regulators include endostatin, platelet factor 4, thrombospondin, plasminogen (angiostatin), high molecular weight kininogen (domain 5), and α -2 antiplasmin, among others. These stimulators and inhibitors of endothelial cell migration and proliferation may be released into a blood clot in a wound in an orchestrated sequence to regulate neovascularization.⁴⁴² For example, thrombin induces the release of the proangiogenic protein VEGF from platelets.³⁵⁶ Thrombin also induces the release of endostatin from platelets.⁴⁴³

Thrombin induction of endostatin release is mediated by proteinase-activated receptor-4 (PAR-4), a mechanism that is independent of platelet aggregation and of the action of ADP. When a clot forms in a wound,

invasion by microvascular endothelial cells is initially inhibited, which avoids premature clot lysis.⁴⁴² Subsequently, endothelial cells are recruited into the clot during its neovascularization ("granulation tissue"). It is not known how the release of negative and positive regulators of endothelial migration from platelets is orchestrated in a wound. Recent evidence, however, reveals that if a thrombus in a large vessel is recanalized, the neovasculature is made up mainly of bone marrow-derived endothelial cells.⁴⁴⁴ This is analogous to certain tumors (eg, lymphomas) that recruit the majority of their endothelial cells from bone marrow. Endostatin is also released from platelets by certain drugs, such as ticlopidine, which inhibits platelet aggregation by blocking the action of ADP with its receptor.⁴⁴⁵

The nonsteroidal antiinflammatory drugs celecoxib and flurbiprofen or HCT-1026 (a nitric oxide-releasing derivative of flurbiprofen), all of which are cyclooxygenase inhibitors, also release endostatin from platelets and elevate the plasma level of endostatin.⁴⁴⁶ From these findings, one can ask whether the antiangiogenic activity of celecoxib (Celebrex) operates in part by releasing endostatin from its stores and making it available to endothelial cells in an angiogenic site. Also, future research will be necessary to answer other questions. What is the turnover rate of endostatin in platelets? Do cancer patients on long-term celecoxib therapy deplete their platelet stores of endostatin? Can platelets be loaded with endostatin that is administered systemically or in vitro? Would cancer patients treated with celecoxib benefit from periodic endostatin therapy? Would patients on long-term endostatin benefit from celecoxib therapy? Taken together these findings indicate that endostatin is normally distributed among three compartments: (1) stored as a cryptic fragment in collagen XVIII in basement membranes; (2) stored in platelets; and (3) circulating as low levels of free endostatin. A fourth compartment can be considered as the concentration of endostatin in and around angiogenic vessels in tumors, and possibly at other angiogenic sites.

Control of vascular permeability

Endostatin decreases vascular permeability. bFGF treatment increases permeability of [³H]inulin through confluent monolayers of capillary endothelial cells in a transwell assay, an effect that is counteracted by co-treatment with endostatin.⁴²¹ In mice that have received an intravenous injection of Evans blue dye, a large blue stain appears at the site of a subcutaneous injection of VEGF or platelet-activating factor (PAF) (Miles test). Pretreatment with endostatin blocks the permeability induced by these proteins (S. Soker, J. Heymach, and J. Folkman, unpublished data). These results imply that systemic administration of endostatin to patients may decrease edema in brain tumors, but this speculation has not yet been tested. **↑ TOP**

Experimental therapeutics including gene therapy

Insoluble protein administered systemically

Systemic administration of endostatin can inhibit or regress different

tumors. The first recombinant endostatin was from *Escherichia coli* in an insoluble form, because this had previously been successful with angiostatin, and because refolding of the inclusion body to a soluble form by urea, guanidine-HCL and glutathione gave a low yield (1%).^{65, 67, 69} When this endostatin preparation was administered subcutaneously every day for prolonged periods of time in mice (185 days for Lewis lung carcinoma, 160 days for T241 fibrosarcoma, and 80 days for B-16 melanoma), there was no drug resistance. After discontinuation of endostatin at these time periods, tumors did not recur. They remained dormant at a microscopic size.⁶⁵ This was not due to an immunologic mechanism. Wounding the tumor site or moving the tumor to another site in the same mouse initiated tumor neovascularization and growth. Another possibility is preferential storage of endostatin in the matrix at the site of the regressed tumor.⁴⁴⁸ We have observed this same dormancy after 40 days of endostatin therapy of rat mammary cancer induced by an oral carcinogen.⁴⁴⁹ Other investigators have reported successful tumor inhibition with insoluble endostatin from *E. coli*.⁴⁵⁰ Also, soluble endostatin from *E. coli* has inhibited tumor growth.⁴⁵¹ [top](#)

Soluble recombinant endostatin

A wide variety of other tumors in many different laboratories have been inhibited by endostatin in mice and rats without evidence of toxicity or drug resistance. These include lung adenocarcinoma, thyroid carcinoma, colon carcinoma, leukemia, human nonsmall cell lung cancer, human pancreatic cancer, human neuroblastoma, mammary cancer (soluble endostatin from *E. coli*), colon cancer metastases to liver, spontaneous mouse mammary carcinoma (delayed onset, decreased tumor burden, and prolonged survival), and spontaneous pancreatic islet carcinomas.^{72,452-460} Mammary carcinomas were more potently inhibited by a mutated form of endostatin in which an alanine residue was substituted for a proline at position 125 (P125A), than by the wild-type endostatin.⁴⁵⁸ Gliosarcoma both orthotopic and ectopic in rats was markedly inhibited (~50%) after only 10 days of treatment by a relatively low dose of murine endostatin (0.3 mg/kg).⁴⁶¹ Inhibition of tumor growth persisted after cessation of therapy. Yamaguchi and colleagues reported that endostatin administered into a tumor will inhibit tumor growth at significantly lower doses than systemic administration.^{461a} [top](#)

Bolus versus continuous administration

A general characteristic of optimal antiangiogenic therapy is that angiogenesis inhibitors should be at effective levels in the blood continuously. Endothelial cells in the vascular bed of a tumor are constantly exposed to a proangiogenic stimulus from the tumor cells and local stroma. This stimulus needs to be continuously opposed by inhibitors in the blood. An analogy would be titration of blood glucose by insulin, where endothelium is glucose. In a compelling experiment, 24-hour continuous parenteral administration of human endostatin was 10-fold more effective than the same dose administered as a bolus once every day, against human pancreatic cancer in SCID immunodeficient mice (se

Figure 11-1).⁷² Tumor growth was inhibited in a dose-dependent manner, but only continuous administration caused tumor regression. It should be noted that continuous administration of endostatin in vitro (by a floating micro-osmotic pump in 24-well plates) inhibited endothelial proliferation more significantly than once daily addition of endostatin. [↑ TOP](#)

Combinations of endostatin with angiostatin

Reports of endostatin administered in combination with angiostatin, whether as separate proteins, separate genes, or as fusion protein of angiostatin and endostatin in tumor-bearing animals, all show a synergistic increase in efficacy.^{69, 386, 375, 462} While the mechanism of synergy is unclear at this writing, one possibility is that endostatin and angiostatin each up-regulate thrombospondin expression or protect it from enzymatic destruction (Figure 11-13). [↑ TOP](#)

Combination of endostatin with chemotherapy, radiotherapy, or immunotherapy

Endostatin (and angiostatin) potentiate radiotherapy in tumor-bearing mice, when the inhibitor is administered at the same time as the ionizing radiation.⁴⁶³ Tumors underwent regression when treated with a combination of ionizing radiation and endostatin, but not with either agent alone, even when the endostatin dose was lowered below the effective dose and the tumor cells were radioresistant. It can be speculated that radiation damage to active endothelium in a tumor bed may not be repaired in the presence of an angiogenesis inhibitor. This experiment also suggests that radiotherapy may be in part endothelial-dependent. The optimal schedule of radiotherapy and antiangiogenic therapy for cancer patients is unclear.


Endostatin may also potentiate immunotherapy. In an elegant experiment by Davidoff et al the gene for the potent immunogen, green fluorescent protein (GFP) and the gene for endostatin were transferred together to murine neuroblastoma cells prior to inoculation into syngeneic immunocompetent mice.⁴⁶⁴ Prevention of formation of appreciable tumors was effected in 63% of mice, while tumor cells expressing endostatin alone or GFP alone yielded only a modest delay in tumor growth. Mice treated with the combination were protected against subsequent tumor challenge with unmodified cells. Endostatin also potentiates conventional chemotherapy in tumorbearing animals.⁴⁶⁵ However, it is not clear how combinations of antiangiogenic therapy and chemotherapy at maximum tolerated dose schedules will translate to patients (see below). [↑ TOP](#)

Endostatin gene therapy


Endostatin gene therapy in tumor-bearing mice has generally been successful when administered locally, but it has been problematic when administered systemically.

Local gene therapy.

In an example of local gene therapy, mouse liver tumor cells transfected with a retroviral vector containing the murine endostatin gene were injected subcutaneously or into the peritoneal cavity.⁴⁶⁷ After 63 days, control tumors (vector without endostatin) reached 2400 mm³ compared to 30 mm³ for endostatin transduced tumor cells ($p < 0.001$). After 123 days all 16 mice given an intraperitoneal injection of control tumor cells had died, compared to only 3 of 32 mice given an injection of the endostatin tumor cells (91% survival) (Figure 11-14).⁴⁶⁷ Increased levels of endostatin were detected in tumor lysates, but not in serum of mice with subcutaneous tumors. Other reports of local gene therapy with endostatin show effective tumor inhibition in animals. Examples are inhibition of human liver carcinoma, renal cell carcinoma, mouse melanoma.⁴⁶⁸⁻⁴⁷¹ A human breast carcinoma, MDA-MB435, was markedly inhibited when the cells were transduced with endostatin, but another breast cancer MCF7 was not inhibited.³⁶⁵ (However, angiostatin gene transfer inhibited MCF7, but not MDA MB435).³⁶⁵ Gliomas were inhibited by gene transfers to tumor cells that were then implanted into rat brains.⁴⁷² Gliomas were also inhibited by intratumoral implantation of alginate-encapsulated beads containing packaging cells transfected with endostatin.⁴⁷³⁻⁴⁷⁵

Of interest is that intratumoral gene transfer of endostatin inhibited mouse mammary tumors (MidT2-1), but had a minimal effect in human breast MDA-MD-231 in SCID mice.⁴⁷⁶ Despite many successes of endostatin gene transfer into tumor cells, unsolved problems remain. The fact that one author can report marked and sustained tumor inhibition with endostatin transduction of a mouse breast cancer, but minimal effect in a human breast cancer MDA-MD-231, highlights the problem.⁴⁷⁶ A third author reports marked inhibition by endostatin gene transfer into a human breast cancer MDA-MBA335, but minimal effect in MCF7, yet angiostatin gene transfer into MCF7 was very inhibitory, but had only minimal effect on MDA-MB435 breast cancer cells.³⁶⁵ For unknown reasons, the inhibitor may lose activity when produced by one type of tumor cell, but not by another. Whether this is a protein-folding problem, or results from excess local levels of endostatin at the endothelial receptor site is not clear (O. Kisker, et al., unpublished data). A fourth author reports that breast cancer (MCA-4) transduced with endostatin is significantly inhibited.⁴⁷⁷  TOP

Systemically administered gene therapy.

Systemically administered gene therapy (by an intravenous or intramuscular vector in mice or rats) significantly inhibited tumor growth in renal cell carcinoma, Lewis lung carcinoma adenocarcinoma MC38, hemangioendothelioma, spontaneous breast cancer and its metastases, and colon cancer (when the gene was injected intramuscularly and endostatin serum levels were only moderately elevated, [35–40 ng/mL]).^{367, 469, 478-481} Adenoviral gene transfer of endostatin delayed growth of breast cancer implants and retarded their growth by 67% after the tumors appeared.^{478a}  TOP

Problems with systemic gene therapy.

However, other reports of systemically administered endostatin gene therapy failed to inhibit tumor growth. For example, either local or systemic endostatin gene therapy failed to inhibit rat tumors despite the achievement of serum levels more than 10 to 200 times above normal levels of endostatin (eg, 500 ng/mL up to 10 μ m/mL).⁴⁸² However, these tumors were significantly suppressed when an inhibitor of plasmin was administered together with endostatin by gene therapy, but not when the plasmin inhibitor was administered alone.⁴⁸² This result suggests that very high levels of plasmin in a tumor bed may interfere with endostatin activity. It is interesting that angiostatin, by binding to annexin II when it is overexpressed by endothelial cells in an angiogenic site, decreases plasmin production on the endothelial surface.^{326,338–341}

It remains to be demonstrated if this mechanism plays a role in the synergy of angiostatin-endostatin combinations.^{69, 375, 386, 462} We also were unable to demonstrate significant inhibition of tumor growth and only 23% to 33% ($p < 0.002$ and 0.0001 respectively) inhibition of corneal angiogenesis, when viruses encoding angiostatin or endostatin were administered as a single intravenous injection, despite the fact that peak serum levels of endostatin reached 400 to 599 times above the normal level.⁴⁸⁵ In contrast, viruses encoding soluble forms of Flk1 or Flt1, the receptors for VEGF, resulted in approximately 80% growth inhibition of the same tumor and strong inhibition of corneal neovascularization (74% to 80%) ($p < 0.001$). A strong correlation was observed between the effects of the different viruses on tumor growth and the activity of the viruses in the inhibition of corneal micropocket angiogenesis. It is unclear why angiostatin and endostatin delivered by systemically administered gene therapy should be so ineffective as tumor inhibitors, when soluble VEGF receptors administered by the same viruses and in the same tumor models should inhibit tumor growth so effectively. However, three speculations may be considered as a basis for future research. Antiangiogenic and antitumor activity of endostatin are dose-dependent, but recent experiments reveal a U-shaped curve (O. Kisker, et al., unpublished data), analogous to interferon- α and also TGF- β .^{485a, 485b} In one tumor system human recombinant endostatin protein administered at 50 mg/kg/day inhibited tumor growth by 47%; 100 mg/kg by 84% and 1000 mg/kg by 37%. Therefore, it is possible that super high levels of endostatin above an optimum level lose activity in vivo. A second speculation is that endostatin may aggregate at very high serum levels, or that its folding may be affected. A third speculation is that serum plasmin levels may be increased by certain viruses or by high viral titers. Other authors subsequently have reported failure of endostatin gene transfer to significantly inhibit a murine leukemia, when endostatin was transduced into hematopoietic stem cells and serum endostatin levels of 300 ng/mL were reached, or to inhibit metastases from a fibrosarcoma when serum endostatin levels of up to 700 ng/mL were obtained.^{483, 484} Of interest is that these authors previously reported in collaboration with Folkman and O'Reilly, enhanced antitumor activity with an endostatin-angiostatin fusion protein engineered in hematopoietic stem cells.^{484, 484a} [top](#)

Endostatin signaling network

To determine how the endothelial cell genome and proteome react to endostatin treatment, we used a genome-wide expression profile covering 95% of the human genome and phosphorylation analysis on antibody arrays (Abdollahi A, Maercker C, Rastert R, et al. Endostatin signaling network. [In preparation]) Briefly, we generated the Human Unigene Chip II containing 74,834 elements, which represents one of the largest gene sets interrogated in array-based gene expression studies to date. Alteration in gene expression was analyzed after four-hour treatment human microvascular endothelial cells with human recombinant endostatin (*Pichia Pastoris*, endotoxin-free EntreMed/Calbiochem). Using 12 reusable chips more than 1,800,000 gene expression measurements in total were performed on endostatin-treated endothelium and compared to control samples of untreated endothelium from two different human subjects. The identities of 50,000 gene clusters (spotted DNA clones) have been sequence verified. By these criteria, 429 antigene clusters were more than 5-fold upregulated. In contrast, 421 antigene clusters were downregulated by 80%.

Overexpressed genes during endostatin treatment included apoptosis genes, cardiovascular and stress response genes, (heat shock, etc.), and genes associated with angiogenesis inhibition. Underexpressed genes during endostatin treatment included gene families associated with cancer and angiogenesis induction (the angiogenic switch), as well as transcripts encoding cytokines, cytokine receptors, cellular adhesion molecules, G proteins, G protein-coupled-receptors and transcription factors.

Endostatin downregulated expression of hypoxia-inducible transcription factor-1 α (HIF-1 α) by 2.5-fold. ^{365, 427} Endostatin also downregulated many upstream activators of HIF-1 α , including c-jun/Fos, Ets, and EGFR. On the other side, HIF-1 α normally helps to trigger a coordinated angiogenic response by inducing the expression of genes involved in blood vessel growth including, VEGF and its receptors, VEGFR2, and neuropilin, as well as nitric oxide synthase and cyclooxygenase-2.


Endostatin also downregulated the Id transcription factors. These genes are proangiogenic. Endostatin substantially downregulated MMP-2 and $\alpha_v\beta_3$ -integrin. ⁴²⁵ Furthermore, Id-1 regulates angiogenesis through transcriptional repression of thrombospondin-1. ⁵²⁸ Therefore, endostatin, by downregulating Id-1, up-regulates expression of thrombospondin-1, a powerful angiogenesis inhibitor on its own (see [Figure 11-13](#)).

Ephrin signaling is downregulated by endostatin. The ephrin/Eph family has recently been shown to participate in the regulation of angiogenesis. Both ephrin B1 and ephrin B2 induce sprouting angiogenesis in vitro to an extent similar to the angiogenic activity of VEGF or angiopoietin 1. ^{529, 530}

Endostatin inhibited tumor necrosis factor- α -induced angiogenic signaling by downregulating the TNF- α receptor. Additionally, NF- κ B (nuclear factor of kappa light chain gene enhancer in B-cells) was downregulated

by endostatin. It is thought that activation of NF-kappaB by TNF- α , ionizing radiation or chemotherapeutic agents protects cells from apoptosis. ^{532, 533} Therefore, endostatin inhibition of NF-kappaB in these settings should enhance apoptosis of endothelial cells. STAT1 and STAT3 were downregulated in endothelial cells treated by endostatin. STATs (signal transducers and activators of transcription factors) up-regulate genes that encode inhibitors of apoptosis (Bcl-family), inducers of angiogenesis (VEGF), and cell cycle regulators (cyclins D1/D2 and c-myc). ⁵³⁴ After endostatin treatment of endothelial cells, most of the genes downstream of STAT were strongly downregulated. These include the apoptosis inhibitor Bcl-2, and the cell cycle regulators, cyclins D1/D2 and c-myc.

Thrombin receptors (PAR-1 and PAR-2) were also significantly downregulated by endostatin. Thrombin is a potent stimulus for release of VEGF by transcriptional regulation activation of HIF-1 α . ⁵³⁵ Thrombin also up-regulates expression of VEGF receptors on endothelial cells. ⁵³⁶ In a positive loop VEGF accelerates thrombin generation, another proangiogenic event. ⁵³⁷ This loop has implications for cancer patients. The majority of cancer deaths are directly attributable to thrombosis or bleeding. This observation emphasizes that endostatin downregulates proangiogenic genes and simultaneously up-regulates genes that lead to inhibition of angiogenesis.

Endostatin directly downregulated Ets-1 in endothelial cells in a time-dependent manner and decreased the mRNA level of most of the Ets-1 target genes like MMP-1 and integrin- β 3. Ets positively regulates angiogenesis by inducing several target genes including uPA, MMP-1, MMP-3, MMP-9 and integrin- β 3, and the elimination of the Ets-1 activity by a dominant negative molecule inhibits angiogenesis in vivo. ⁵³⁸⁻⁵⁴² A striking finding is that several pathways thought to be distinct, in fact intercommunicate to bring about a focused antiangiogenic response in the tumor bed, directly as a result of endostatin treatment. It is not clear whether many of the small molecular angiogenesis inhibitors produce such a orchestrated antiangiogenic response at the genetic level as endostatin does.  **TOP**

Clinical applications of endostatin

There are several interesting characteristics of endostatin that make it advantageous for clinical use in cancer patients. First, it is not toxic. ²⁶⁷ At this writing, endostatin is in phase I/II clinical trials, and to date it has only been used in not more than approximately 120 patients. However, all centers report that there are virtually no side effects, even in patients who have been on endostatin for up to one year (Endostatin Banbury Conference. A critical review of endostatin and its biology; 2002 Mar 10-13; Cold Spring Harbor Laboratories, New York). Patients who receive the subcutaneous preparation of endostatin take their own injections each day at home, like insulin.

Wound Healing.

Endostatin does not delay wound healing in patients, nor does it delay wound healing in animals. ⁴⁸⁶ We have confirmed that wound healing in mice is not delayed by endostatin doses sufficient to completely inhibit growth of tumors in the same animal (J. Marler, et al., unpublished data). [⬆ TOP](#)

Pregnancy.

Endostatin has no effect on pregnancy in mice. Pregnant mice treated with tumor-regressing doses of endostatin deliver normal babies, even if the mothers bear a tumor that is then inhibited by the endostatin (Robert D'Amato, et al, unpublished data). [⬆ TOP](#)

Down Syndrome.

Serum endostatin levels are significantly elevated in Down syndrome patients. ⁴⁹² Endostatin serum levels in normal individuals range from 4 to 40 ng/mL (20.3 ± 11.5 , mean and SD). For Down syndrome individuals, it is 38.6 ± 20.1 , a significant elevation. The collagen XVIII gene is on chromosome 21q22.3. Because Down syndrome is most commonly caused by a duplication of one of the copies of chromosome 21, these individuals have an extra copy of collagen XVIII in which endostatin is a cleavage product. Down syndrome individuals have a low incidence of solid tumors.

A recent study in the United States of 17,987 people with Down syndrome revealed that they have approximately 0.1 the incidence of the control age-matched population for virtually all malignancies except testicular cancer and leukemia. ⁴⁹³ This low incidence of cancer is associated with a large reduction in cancer associated deaths among Down syndrome patients. It has been proposed that an increase of about one-third of normal endostatin serum levels may represent an effective therapeutic dose to significantly inhibit many solid tumors. ⁴⁹² When this observation is taken together with endostatin's lack of toxicity, endostatin may someday be used chronically in a preventive mode for patients with a high cancer risk for breast cancer or other tumors. [⬆ TOP](#)

Clinical Trial.

A polymorphism in endostatin predisposes for the development of prostate cancer. ⁴⁹⁴ A single missense substitution of asparagine for aspartic acid at D104N located in the C-terminal globular domain NCI of collagen XVIII, yields endostatin with this mutation at N104. Men who are heterozygous for this mutation have a 2.5 times increased risk of prostate cancer. The authors propose that (1) the DNA segment containing this residue might contain a novel interaction site to an as yet unknown receptor; (2) the presence of N104 may impair the angiogenesis inhibitory function of endostatin; and (3) this mutation could possibly be used for a prostate cancer screening test. ⁴⁹⁴ [⬆ TOP](#)

Prostate Cancer.

In a phase I trial of endostatin 25 patients who had failed all previous therapy were treated with endostatin.²⁶⁷ They received a daily intravenous bolus dose of endostatin over a period of 20 minutes. Endostatin was safe and had a linear pharmacokinetic profile up to 599 mg/m²/day. Tumors regressed in two patients, although not sufficiently to satisfy the criteria for the terms 'partial' or 'complete' response used for chemotherapy. In one patient with metastatic melanoma there was prolonged stabilization for 426 days with a much improved quality of life and he returned to work.

The limited antitumor effect of bolus dosing in these patients is similar to the weak antitumor effect observed in mice that received bolus dosing.⁷² In ongoing phase II trials, patients in Boston are receiving subcutaneous injections of a slow-release preparation of endostatin, which provides a steady state blood level of endostatin similar to that obtained by continuous intravenous dosing in patients and continuous intraperitoneal pump dosing in mice.⁷² In these patients endostatin is showing more anticancer activity and more prolonged stable disease up to 1 year or more. [↑ TOP](#)

Production and manufacture of endostatin

Recombinant endostatin can be produced in large scale in *E. coli*, *Drosophila* cells, *P. pastoris* and in mammalian cells.^{487–491} Current clinical trials in the United States and The Netherlands employ recombinant human endostatin produced in yeast (*P. pastoris*). [↑ TOP](#)

A family of angiogenesis inhibitors in matrix proteins

The discovery of endostatin, a specific and potent angiogenesis inhibitor residing as an internal fragment of collagen XVIII, further implicated the potential role of basement membranes in the regulation of angiogenesis. In 1996, three independent lines of evidence led Kalluri and his colleagues to launch a new line of investigation into the role of vascular basement membrane in the regulation of angiogenesis: (1) the observation that vascular basement membrane proteins are associated with resting and growth arrested capillary endothelial cells, while proliferating and migrating endothelial cells are under the influence of provisional matrix molecules such as vitronectin and fibronectin; (2) studies by Madri and also by Furcht that suggest that sub-domains of type IV collagen exhibit different properties as compared to the intact full-length molecule; and (3) the demonstration by Furcht that the noncollagenous-1 domain (NC1 domain) can disrupt the assembly of type IV collagen.^{498–500,670–673} Kalluri hypothesized that during the inductive phase of angiogenesis involving the degradation of vascular basement membrane, proteolytic degradation fragments of basement membrane are liberated with the potential for positive and negative regulation of capillary endothelial proliferation and migration. Such a potential role for matrix fragments could provide the regulatory balance for an organized formation of new capillaries.

A biochemical approach was designed to extract vascular basement membranes and perform degradation with tumor microenvironment

associated enzymes. The isolated vascular basement membrane per se, did not reveal any antiangiogenic activity in its solid state. But, degradation by various basement membrane-degrading enzymes liberated cryptic domains of collagen molecules from the vascular basement membrane with novel antiangiogenic activity.⁵⁰⁰ Interestingly, while these fragments are present in the intact molecules, in this form they do not exhibit antiangiogenic activity. The initial screen of fragments from vascular basement membrane identified three antiangiogenic fragments from type IV collagen, known as arresten, canstatin and tumstatin. Endostatin was also identified in this collection of basement membrane fragments. The discovery of arresten, canstatin, tumstatin and $\alpha 6$ chain NCI domain of type IV collagen was later also independently published by Petitsclerc and coworkers.⁵⁰⁴

Arresten

In vitro antiangiogenic activity

Arresten (26 kDa) is the NCI domain fragment of $\alpha 1$ type IV collagen.²⁶² Arrestin exhibits antiangiogenic activity in various in vitro and in vivo assays and is found circulating in normal individuals. Recombinant arresten inhibited the growth of small and large renal cell carcinoma xenografts and prostate carcinoma-xenografts in nude mice at doses as low as 4 mg/kg/day/ intraperitoneally. The decrease in tumor size was consistent with a decrease in CD31 positive vasculature.²⁶² [↗ TOP](#)

Mechanism of action.

The antiangiogenic and antitumor action of arresten is potentially mediated by heparan sulfate proteoglycan and $\alpha_1\beta_1$ -integrin on proliferating endothelial cells.²⁶² Two independent studies support the contention that arresten may function via $\alpha_1\beta_1$ -integrin. Senger and colleagues demonstrate that α_1 -integrin-neutralizing antibodies can inhibit VEGF driven angiogenesis without detectable effect on pre-existing vasculature, and VEGF can induce expression of $\alpha_1\beta_1$ - and $\alpha_2\beta_1$ -integrins.⁵⁰¹ Additionally, Pozzi and colleagues demonstrate that tumors grow slower in α_1 integrin null mice, when compared to the wild-type mice.^{502, 503} Collectively, all of these studies suggest that arresten can potentially act as an endogenous inhibitor of $\alpha_1\beta_1$ -induced tumor angiogenesis. [↗ TOP](#)

Canstatin

Canstatin (24 kDa) is the NCI domain fragment of $\alpha 2$ type IV collagen.²⁶³ Canstatin exhibits antiangiogenic activity in several in vitro and in vivo assays.²⁶³ Recombinant canstatin inhibits the growth of small and large renal cell carcinoma tumors by four- or three-fold with respect to placebo-treated mice. Established human prostate carcinoma (PC-3) in severe combined immunodeficient (SCID) mice or athymic (nu/nu) mice

exhibited fractional tumor volumes of about threefold less than placebo-treated mice when treated with 3 mg/kg canstatin. This decrease in tumor size was consistent with a decrease in CD31 positive vasculature.²⁶³ Recent studies using syngeneic mouse Lewis lung tumors (LLC) in C57BL/6 mice and orthotopic MDA-MB 435 breast tumors in nude mice also reveal significant tumor inhibition with recombinant canstatin. The antiangiogenic activity of canstatin was also independently validated subsequently by Petitclerc and coworkers.⁵⁰⁴ Petitclerc and colleagues demonstrate that recombinant canstatin, tumstatin, and the $\alpha 6(\text{IV})\text{NC1}$ domain can inhibit angiogenesis in a CAM assay. Petitclerc and colleagues also show that canstatin can bind to $\alpha_v\beta_3$ -integrin on endothelial cells.

Mechanism of action.

The mechanism of how canstatin inhibits proliferation and migration of endothelial cells was further investigated. Kamphaus and colleagues investigated the effect of canstatin on ERK activation induced by 20% fetal bovine serum and endothelial mitogens. Canstatin does not act primarily by inhibiting VEGF or bFGF activation of ERK.²⁶³ Instead, canstatin specifically induces apoptosis of endothelial cells with insignificant effect on nonendothelial cells. Apoptosis of endothelial cells by canstatin is mediated by induction of a steady decrease in FLIP protein (an antiapoptotic protein associated with FAS-mediated apoptosis pathway) levels in the presence of 20% fetal calf serum, bFGF and VEGF. Interestingly, canstatin did not affect FLIP levels in the absence of growth factors. Canstatin's lack of effect on endothelial cells in the absence of growth factors may indicate that only proliferative endothelium is targeted.²⁶² Recent studies suggest that canstatin binds to $\alpha_v\beta_3$ -integrin and also $\alpha_3\beta_1$ -integrin on proliferating endothelial cells.⁵⁰⁴ The importance of $\alpha_v\beta_3$ and $\alpha_3\beta_1$ -integrins for the antiangiogenic activity of canstatin is yet to be determined. [↑ TOP](#)

Tumstatin

Tumstatin (28 kDa) is the NC1 domain fragment of $\alpha 3$ collagen molecule and exhibits antiangiogenic activity in both the in vitro and in vivo assays.^{71, 264, 505, 506, 507} The antiangiogenic activity has been narrowed down to a 25 amino acid stretch in the middle of the molecule. At equal molar concentrations, the full-length tumstatin exhibits similar activity as the 25 amino acid peptide termed as T7 peptide^{71, 264, 505, 506, 507} Recombinant tumstatin and T7 peptide exhibit significant inhibition of human tumor xenografts in nude and SCID mice, syngeneic LLC and B16F10 tumors in C57BL/6 mice, and orthotopic MDA-MB 435-breast tumor in nude mice at a dosage of 1 mg/kg/day/ip. or iv.

Tumstatin ($\alpha 3(\text{IV})\text{NC1}$) binds to endothelial cells via $\alpha_v\beta_3$ -integrin and $\alpha_6\beta_1$ -integrin.^{71, 264, 505, 506} The $\alpha_3\beta_1$ -binding activity for tumstatin was also demonstrated by Petitclerc and colleagues.⁵⁰⁴ We show that the

binding to $\alpha_v\beta_3$ is pivotal for the antiangiogenic activity associated with tumstatin and that the activity is restricted to amino acids 54–132 (tum-5) within the 244 amino acid tumstatin using deletion mutagenesis. ^{71, 264, 506} Additionally, $\alpha_v\beta_3$ binding to tumstatin is mediated via a mechanism independent of the RGD-containing amino acid sequence and vitronectin/fibronectin binding of proliferating endothelial cells. The antiangiogenic activity of tumstatin can be further localized to a 25 amino acid region within tum-5 and a synthetic peptide representing this region preserves 'full' antiangiogenic activity and binds to $\alpha_v\beta_3$ -integrin. ^{71, 264, 506} Cell biological experiments demonstrate that the antiangiogenic activity of tumstatin is dependent on $\alpha_v\beta_3$ -binding on proliferating endothelial cells. ^{71, 506} These experiments support the notion that through the action of endogenous inhibitors such as tumstatin, $\alpha_v\beta_3$ -integrin could also function as a negative regulator of angiogenesis. ^{71, 508–511}

Mechanism of Action.

Tumstatin induces apoptosis of proliferating endothelial cells. ⁵⁰⁷ Maeshima and colleagues demonstrate that tumstatin is an $\alpha_v\beta_3$ -integrin-dependent inhibitor of cap-dependent translation mediated through negative regulation of mTOR signaling. In essence, tumstatin is an endothelial cell specific inhibitor of cap-dependent translation. The mTOR inhibitory property of tumstatin mimics that of rapamycin, except unlike rapamycin, which is pan-specific, tumstatin is specific for only endothelial cells. ⁵⁰⁷ The action on mTOR is mediated by the FAK-PI3K-Akt pathway downstream of $\alpha_v\beta_3$ -integrin. ♦ TOP

Endogenous angiogenesis inhibitors as cryptic tumor suppressor proteins

With the discovery of angiostatin, endostatin, arresten, canstatin, and tumstatin, a new paradigm has emerged that puts forward a novel notion that some proteins such as plasminogen and basement membrane collagens, harbor unique properties that are cryptic and become exposed only upon proteolytic degradation. Thus, in their intact form (full-length) plasminogen and basement membrane collagen do not exhibit antiangiogenic activity or antitumor activity; but, when plasminogen and basement membrane collagens undergo degradation, they now expose or liberate novel cryptic fragments, which possess antiangiogenic activity.

All of these inhibitors are found in blood of normal individuals. A decrease in the physiologic levels of tumstatin is speculated to result in an acceleration of tumor growth. A change in amino acid sequence of endostatin due to a polymorphism correlates with an increased susceptibility to prostate cancer. Conversely, an increase in physiological levels of endostatin results in less propensity for emergence of solid tumor in Down syndrome patients (due to an extra copy of type XVIII collagen) (see above). ⁴⁹² Collectively, these results argue that angiostatin, endostatin, tumstatin, canstatin and arresten may function as cryptic tumor

suppressor proteins, offering an additional line of defense against tumor progression by blocking angiogenesis switching.

Antiangiogenic conformation of antithrombin III

A human small-cell lung carcinoma suppressed angiogenesis and tumor growth at remote sites in immunodeficient mice. These cells generated an enzyme in vitro that converted the 58 kDa conformation of circulating antithrombin III to a 53 kDa form of the protein in which the externally configured stressed loop of antithrombin was retracted into the body of the molecule.⁷⁰ The 53 kDa "cleaved" form is a specific endothelial inhibitor and a potent angiogenesis inhibitor and has no thrombin binding activity. Antithrombin III has no antiendothelial or antiangiogenic activity. The enzyme(s) that induce this conformational change have not yet been elucidated. Human pancreatic cancer also generates the 53 kDa cleaved antiangiogenic antithrombin.⁵¹² [↑ TOP](#)

Tropinin I

A novel protein, troponin I, was purified from cartilage during an attempt to find the inhibitors responsible for the avascularity of cartilage.⁵¹³ It is a 22 kDa subunit of the troponin complex that, along with tropomyosin, is responsible for the calcium-dependent regulation of striated muscle contraction. It is a specific inhibitor of endothelial cell proliferation and of angiogenesis in the mouse cornea and chick embryo. It inhibits growth of primary tumor and metastases. [↑ TOP](#)

Other angiogenesis inhibitors that are endogenous

In addition to the specific angiogenesis inhibitors, endostatin, tumstatin, canstatin and arresten discussed above, the plasma and tissues contain other endogenous angiogenesis inhibitors that, although not specific, appear to preferentially inhibit endothelial proliferation and/or migration. These inhibitors have quite different structures. The increasing number of endogenous inhibitors of angiogenesis being discovered indicates the existence of a family of molecules that provide physiologic suppression of angiogenesis. An analogy may be drawn to the more than 40 proteins of the clotting system, many of which function to prevent coagulation under normal conditions (see below). Examples of endogenous angiogenesis inhibitors include interferon- β , platelet factor 4, fibronectin fragments, fragments of SPARC, thrombospondin-1, tissue inhibitors of metalloproteinases (TIMPS), certain angiostatic steroids such as tetrahydrocortisol, a nonmineralocorticoid metabolite of cortisol, and 2-methoxyestradiol, and interleukin-12.^{243, 246, 247, 259-261, 514-525} Thrombospondin also inhibits lumen formation, a rather unique property of an angiogenesis inhibitor.⁵²¹ In addition to its cytotoxic properties, mediated by T-cell activation, interleukin-12 also induces the upregulation of interferon- γ , which itself up-regulates inducible protein 10 (IP-10), reported to be an angiogenesis inhibitor.⁵²⁶ Certain cryptic antiangiogenic protein fragments are contained within larger proteins of the hemostatic system, in addition to angiostatin and the cleaved conformation of antithrombin III. These include an internal fragment (domain 5) of high

molecular weight kininogen in plasma and the first kringle domain (NK1), or the first two kringle domains (NK2) of hepatocyte growth factor (HGF), itself a stimulator of angiogenesis in platelets.⁵²⁷ The hemostatic system, like the extracellular matrix, also appears to store certain angiogenesis inhibitors that may be needed during wound healing angiogenesis (for review see Browder, et al.).⁴⁴² [↑ TOP](#)

Synthetic angiogenesis inhibitors

TNP-470

Takeda neoplastic product-470 (TNP-470) is a synthetic analogue of fumagillin, a compound secreted by the fungus *Aspergillus fumigatus fresenius*, which selectively inhibits methionine aminopeptidase-2 and blocks cdk2, inhibits cdc, and inhibits phosphorylation of Rb.^{52, 534, 544} Against animal models of human, rabbit, hamster, rat and mouse tumors, both primary and metastatic, reports from more than 100 laboratories show that TNP-470 has the broadest anticancer spectrum of any known agent.⁶⁰ TNP-470 at 30 mg/kg subcutaneously every other day inhibited the growth of human glioblastomas, embryonal rhabdosarcoma, meduloblastoma, meningioma, fibrosarcoma, neurofibrosarcoma, hemangioendothelioma, and gastric, colon, breast, prostate and esophageal carcinomas, as well as murine Lewis lung carcinoma, melanoma, osteosarcoma, reticulum cell sarcoma, glioma, hepatoma, fibrosarcoma and mammary, bladder, tongue and pancreatic cancer by 50 to 95% with complete regression in a variety of tumors especially those of the central nervous system.

In early clinical trials with TNP-470 used as a single agent, best results were that 18% of patients had a 50% regression of tumor. Furthermore, 5 patients had "complete or dramatic and durable tumor regression, despite having failed all conventional therapy," including patients with carcinoma of the cervix metastatic to lung, high grade sarcoma of the kidney, renal cell carcinoma, androgen-independent prostate cancer, and Kaposi's sarcoma.⁵⁴⁵⁻⁵⁴⁹ TNP-470 also showed promise when used in combination with chemotherapy.⁵⁵⁰⁻⁵⁵² However, at effective therapeutic doses, some patients have experienced neurocognitive toxicity and minor seizures have been reported. Recently, Ronit Satchi-Fainaro in the Folkman laboratory has determined that TNP-470 rapidly enters the cerebrospinal fluid. She has conjugated TNP-470 to a water-soluble synthetic polymer, (2-hydroxypropyl)methacrylamide (HPMA). Lysosomal enzymes (cathepsin B) in activated endothelial cells cleave the linker between the polymer and the drug, releasing free TNP-470. As a result, TNP-470 does not enter the cerebrospinal fluid, the efficacy of the conjugated HPMA-TNP-470 is significantly greater than free TNP-470 in tumor-bearing animals, and a maximum tolerated dose has not yet been found even at 7 times the previous maximum tolerated dose (R. Satchi-Fainaro, Targeting angiogenesis with an HPMA-copolymer-TNP-470 conjugate. [↑ TOP](#)



Thalidomide

In 1994 Robert D'Amato and colleagues reported that the antiinflammatory drug thalidomide could inhibit angiogenesis induced by bFGF or VEGF in a rabbit cornea micropocket assay.²⁴⁹ Thalidomide inhibited new blood vessel formation in rabbits and mice independently of its ability to suppress infiltrating host inflammatory cells.²⁴⁹ Thalidomide reduced the growth of carcinomas in rabbits and Lewis lung carcinoma in mice.⁵⁵⁴ In 1999, thalidomide therapy was shown to be active in humans against advanced multiple myeloma.⁵⁵⁵ Thirty-two percent of patients treated with thalidomide had a positive response, as assessed by reduction of the serum levels of myeloma protein and urine levels of Bence Jones protein. These findings have been confirmed by other studies.⁵⁵⁶ Thalidomide is now being tested in numerous clinical trials in the United States (>160 clinical trials in >70 medical centers in the United States [Celgene Corp., Warren, NJ, January 2001]), and also in Europe for the treatment of a variety of solid tumors and has become one of the most effective drugs for multiple myeloma, either as first line therapy or for the treatment of disease resistant to conventional chemotherapy.

Mechanism of Action.

Thalidomide treatment suppresses production of tumor necrosis factor (TNF)- α , which has been reported to be angiogenic. However, other, more potent inhibitors of TNF- α such as pentoxifylline or dexamethasone have little or no activity against corneal angiogenesis.²⁵² Ibuprofen, which inhibits angiogenesis, actually increases serum levels of TNF- α in mice.²⁵² Furthermore, TNF- α inhibitors are not effective in animal models of myeloma or in patients. TNF- α suppression therefore does not appear to be a major part of thalidomide's antiangiogenic activity.

Thalidomide does have a direct antiproliferative effect on multiple myeloma cells in vitro, although very high concentrations of thalidomide (up to 100 μ M) are required.⁵⁵⁶ Nevertheless, this drug might be able to target both cancer cells and vascular endothelial cells. In patients with multiple myeloma, or myelodysplastic syndromes, plasma levels of the proangiogenic proteins, VEGF and bFGF were significantly decreased compared to pretreatment levels. This decrease correlated with efficacy of thalidomide therapy.¹³⁸ [TOP](#)

Surrogate markers of efficacy in patients.

In the original clinical reports of the antitumor effects of thalidomide against multiple myeloma, decreased microvessel density was not associated with disease remission.⁵⁵⁵ This created a question of whether the clinical efficacy of thalidomide was mediated entirely or even partly by its antiangiogenic features.⁵⁵⁷ The problem is an assumption that because microvessel density (measured by the method of Weidner and colleagues) can determine cancer prognosis, it should also be useful for determining therapeutic efficacy.⁵⁵⁸ While increased microvessel density in bone marrow is associated with relapse in multiple myeloma or with untreated multiple myeloma in virtually all reports to date, microvessel density has not been an effective measure of therapeutic response in many

patients with multiple myeloma. For example, some studies reported that increased microvessel density persists in patients who have undergone a complete response to thalidomide or to stem cell transplantation. ^{555, 559} Microvessel density also remains elevated in patients who have undergone remission after chemotherapy for acute lymphoblastic leukemia. ⁵⁶⁰ Therefore, microvessel density may not be a useful indicator of efficacy in solid tumors, even though it has continued to be a valid prognostic indicator for predicting future mortality and metastasis. ^{37, 252}

As a substitute for microvessel density, other surrogate markers of efficacy of antiangiogenic therapy are being studied. Circulating bone marrow-derived endothelial cells showed a 10-fold reduction and returned to normal values after thalidomide treatment. ¹³⁸ Decreased circulating levels of VEGF correlated with thalidomide's therapeutic efficacy in multiple myeloma and other hematologic diseases. ¹³⁸ However, serum VEGF alone has not been a predictable marker of angiogenesis in other tumors, in part because of the high VEGF content of platelets, and because circulating angiogenesis inhibitors are not taken into account. ³⁵⁸ [↑ TOP](#)

Principles of angiogenesis important for clinical translation

Antiangiogenic chemotherapy

In some clinical trials angiogenesis inhibitors are also being administered in combination with conventional chemotherapy. Chemotherapeutic agents have in fact been shown to have antiangiogenic properties in animal models, in addition to their ability to induce direct cancer cell death. Paclitaxel, which inhibits microtubule polymerization, inhibits vascular endothelial cell proliferation, motility and invasiveness in a dose-dependent manner in vitro and tumor angiogenesis in vivo. ⁵⁶² Secondary effects such as these could contribute to the antitumor efficacy of chemotherapy in vivo and might delay or prevent the acquisition of drug-resistance by cancer cells. [↑ TOP](#)

Antiangiogenic chemotherapy

Browder and colleagues proposed that the traditional dose schedule regimen for chemotherapeutic agents cannot, however, provide the sustained blockade of angiogenesis achieved by angiogenesis inhibitors. ⁵⁶³ This could be because chemotherapy is usually administered at the maximum tolerated dose, followed by a treatment-free interval to allow recovery of bone marrow and gastrointestinal tract cells. During the treatment-free interval, microvascular endothelial cells in the tumor bed can resume their proliferation and support tumor regrowth. ⁵⁶³ Browder and colleagues experimented with changing scheduling and dose of a cytotoxic agent to augment its antiendothelial activity, an approach called 'antiangiogenic chemotherapy' (metronomic therapy).

Antiangiogenic chemotherapy was first shown to be effective in tumor-bearing mice. ⁵⁶³ Administration of cyclophosphamide at more frequent

intervals at an overall lower dose with a brief treatment-free interval induced sustained apoptosis of endothelial cells in the vascular bed of the tumors, and more effectively controlled growth of drug-resistant tumors. This protocol also reduced side-effects and avoided bone marrow suppression. These results indicated that antitumor efficacy of cytotoxic drugs may be improved by changing the schedule and dose to provide optimum cytotoxic targeting of the microvascular endothelial cells in the tumor bed.

These results might also help to explain why some patients who receive long-term maintenance or even palliative chemotherapy have stable disease beyond the time that the tumor would have been expected to develop drug resistance. Patients with slow-growing cancers who are on antiangiogenic scheduling of chemotherapy involving continuous infusion 5-fluorouracil, weekly paclitaxel, or daily oral etoposide have shown an improved outcome despite the fact that in some of these patients the tumors had already become drug resistant to conventional chemotherapy.^{564–571}

Antiangiogenic chemotherapy has also been called 'metronomic' therapy, but the two terms do not have precisely the same meaning. Antiangiogenic chemotherapy signifies that the target of the chemotherapy is microvascular endothelium in the tumor bed.⁵⁷² Metronomic therapy indicates that the schedule of administration is at very regular intervals. [↑ TOP](#)

Combinations of antiangiogenic therapy and standard chemotherapy

Clinical trials of antiangiogenic chemotherapy are underway at present.^{573, 574} These trials usually combine very low-dose chemotherapy at frequent intervals with one or more angiogenesis inhibitors. For example, a current clinical trial at the Dana Farber Cancer Center, developed by Mark Kieran, combines celecoxib, very low-dose cyclophosphamide, and thalidomide. Every 3 weeks, low-dose etoposide is substituted for cyclophosphamide. In other clinical trials, angiogenesis inhibitors have been added to high-dose conventional chemotherapy. Millar however, has cautioned about possible limitations of combining standard chemotherapy with antiangiogenic therapy or, alternatively, with an oncogene signaling inhibitor that has antiangiogenic activity.⁴⁶⁶ Unexpected toxicities may occur, or at the least, lack of increased efficacy. However, we and subsequently others have reported that angiogenesis inhibitors can be combined with chemotherapeutic drugs which are administered on an 'antiangiogenic' or 'metronomic' dose and schedule; ie, that is more frequently than most conventional schedules, and at significantly lower than maximum tolerated doses.^{37, 563, 572, 575–577} Administration of chemotherapeutic drugs in this manner is designed to maximize their antiangiogenic activity while reducing the severe and acute toxicities often associated with conventional regimens of maximum tolerated dose.⁵⁷⁸ Clinical trials of antiangiogenic chemotherapy (metronomic) are currently underway.^{573, 574} [↑ TOP](#)

Clinical patterns of metastasis may be governed by angiogenic mechanisms

Metastases may present at least four common clinical patterns (see [Table 11-1](#)): (1) a primary tumor such as a colon carcinoma is removed, but within a few months metastases appear; (2) metastases are already present when the primary tumor is first detected; (3) metastases appear first, and the primary remains occult; and (4) the primary is removed (or treated by other therapy), and metastases do not appear until years later (eg, 5–10 years). A fifth and rare pattern is that metastases disappear after removal of the primary tumor (eg, a few cases of renal cell carcinoma). These patterns of metastatic presentation are well recognized, but their biologic basis has been poorly understood.

New experimental evidence suggests that the majority of the presenting patterns of metastases may be dictated by the intensity of angiogenesis in their vascular bed. The essential role that angiogenesis plays in the metastatic cascade can be appreciated by examining animal models that have been developed for each of the common presenting patterns of metastases in cancer patients (see [Table 11-1](#)). These five patterns can be explained by a unifying angiogenic mechanism and will depend mainly upon whether or not a primary tumor generates an angiogenesis inhibitor (s) (eg, angiostatin, endostatin), which circulate and suppress angiogenesis in remote metastases. [97](#) [↑ TOP](#)

Hematologic malignancies are angiogenic

Although solid tumors are known to be angiogenesis dependent, it was assumed until 1993 that leukemias and other hematologic malignancies did not induce angiogenesis to promote their own survival. [579](#) In 1993, Brunner and colleagues observed that bFGF was expressed by human bone marrow and peripheral blood cells, and the following year Nguyen and coworkers reported that bFGF was elevated in the urine of newly diagnosed leukemic patients to higher levels than most other malignancies. [170](#), [580](#) Bone marrow angiogenesis was subsequently found to correlate with multiple myeloma progression, and was also observed in lymph nodes of B cell non-Hodgkin lymphoma patients and in bone marrow biopsies from children with newly diagnosed untreated acute lymphoblastic leukemia. [561](#), [581](#)–[584](#) By 1999, cellular levels of another angiogenic protein, VEGF, were reported to predict outcome of patients with multiple myeloma ([Figure 11-15](#)). [585](#)

Angiogenesis inhibitors might therefore be useful in treating hematologic malignancies. A recent study reports that the retroviral gene transfer of a vector encoding the direct angiogenesis inhibitors angiostatin and endostatin inhibits bone marrow angiogenesis and tumor growth in a mouse model of leukemia. [375](#) This therapy was shown to directly inhibit endothelial proliferation in vitro, but to have no effect on leukemia cell proliferation. Mice inoculated with B-cell, T-cell or myelogenous leukemias and treated with recombinant endostatin have also been observed to live significantly longer and experience fewer toxic side effects than with conventional chemotherapy (T. Browder, et al.,

unpublished observation). [↑ TOP](#)

Host angiogenic response may be genetically controlled

A recent finding is that different strains of inbred mice have an approximately 10-fold range of response to a constant dose of angiogenic stimulation (bFGF) in the corneal micropocket assay. ⁵⁸⁶ Furthermore, the in vitro migratory activity of endothelial cells from aortic rings of selected strains correlates with the in vivo responsiveness. Also, high angiogenesis responders require higher doses of an angiogenesis inhibitor to achieve the same suppression of angiogenesis as a low dose of inhibitor in a low angiogenesis responder. If this early work translates to humans, one can speculate that a low angiogenic host response would decrease the probability of in situ carcinomas switching to the angiogenic phenotype, that tumors that did become angiogenic would grow slowly or be indolent (eg, indolent prostate cancer) and that relatively low doses of angiogenesis inhibitors would be necessary to achieve effective therapy. In contrast, a high angiogenic host response would predict a higher frequency of in situ switching to the angiogenic phenotype, faster growing tumors, and significantly higher doses of antiangiogenic therapy would be required. [↑ TOP](#)

p53 normally suppresses angiogenesis

p53 mutations also allow tumor cells to become resistant to apoptosis under conditions of hypoxia. ⁵⁸⁷ In mice, p53 mutations decrease the response of cancer cells to antiangiogenic therapies. ⁵⁸⁸ Wild-type p53 normally suppresses tumor angiogenesis by upregulating thrombospondin-1, inducing degradation of HIF-1 α , suppressing transcription of VEGF, and downregulating bFGF-binding protein expression. ^{75, 161, 233} The increased neovascularization that occurs following loss of p53 function, however, can be overcome by increasing the dose of antiangiogenic therapy. For example, in mice bearing a mutant p53-associated human pancreatic tumor, there was a dose-dependent response to a single angiogenesis inhibitor, varying from 33% inhibition to 97% inhibition of tumor growth with tumor regression. ^{72, 589}

It remains to be seen whether this result can be translated to those patients with tumors that possess p53 mutations. Therefore, in contrast to the maximum tolerated doses of chemotherapy, antiangiogenic therapy dosing may be optimized by titrating against the total angiogenic output of a tumor, analogous to the titration of insulin against blood sugar or Coumadin against prothrombin levels. There is currently, however, no quantitative method for determining the total angiogenic output of a patient's tumor burden, so surrogate markers such as circulating progenitor endothelial cells are being studied. ¹³⁷ [↑ TOP](#)

Slowly growing indolent tumors

Rapidly growing tumors are generally more sensitive to conventional cytotoxic chemotherapy than slowly growing, indolent tumors. In fact, the most slowly growing tumors (such as neurofibromas and indolent prostate

carcinomas) are virtually unresponsive to chemotherapy. It has been assumed by some oncologists that slowly growing tumors would be as unresponsive to antiangiogenic therapy as they are to chemotherapy. ⁵⁹⁰ However, when two types of human bladder cancers, a rapidly growing highly vascularized tumor and a slowly growing poorly vascularized tumor were implanted into immunodeficient mice, growth of both tumor types was inhibited by angiogenesis inhibitors. For a given dose of angiogenesis inhibitor, the slower the tumor was growing, the more effective the inhibitor. ⁵⁹⁰ In carcinogen-treated mice, breast cancers that arose spontaneously and grew very slowly were inhibited and/or regressed by treatment with mouse endostatin. ²⁸⁵ Spontaneously arising carcinomas of the pancreatic beta cells in transgenic mice grew very slowly compared to most transplantable tumors, but retained a high degree of sensitivity to angiogenesis inhibitors. ⁴⁴⁹ [↑ TOP](#)



Poorly vascularized tumors

Another common misconception about antiangiogenic therapy is that it is only effective in highly vascularized tumors. Some cancer patients who have failed conventional therapy lament that their physician told them they were not candidates for antiangiogenic therapy because their tumor was not vascularized. It is sometimes assumed by surgeons that a tumor that appears to be white and avascular (eg, pancreatic cancer) is not a candidate for antiangiogenic therapy. Based on these assumptions, some angiogenesis inhibitor clinical trials currently require a pretreatment biopsy for microvessel density to exclude patients whose tumor microvessel density is too low. Another clinical trial for a new angiogenesis inhibitor is restricted to renal cell carcinoma and other highly vascularized tumors.

There are several flaws in these assumptions. First, virtually any tumor that is large enough to be visible or palpable has already undergone neovascularization to attain that size. Second, intensity of neovascularization cannot be determined by gross inspection of a tumor. A white neurofibrosarcoma of one or more cubic centimeters may have a microvessel density by microscopy that is similar to the microvessel density of a reddish hepatic carcinoma of similar size, except in the whitish tumor, the vessels are more compressed. Third, the lower the vascularity of a tumor, the more susceptible it appears to be to antiangiogenic therapy. ⁵⁹⁰ Highly vascularized tumors may require higher doses of an angiogenesis inhibitor or combinations of angiogenesis inhibitors to achieve a tumor response. [↑ TOP](#)

Predicting efficacy of antiangiogenic therapy: is microvessel density helpful?

In 1972, a quantitative method for histologic grading of tumor angiogenesis was developed that correlated the amount of neovascularization with tumor grade in human brain tumors. ⁵⁹¹ Additional methods for quantifying grade of tumor vascularization were developed, and followed by the first report of the use of tumor vascularity as a prognostic marker for cutaneous melanoma. ^{103, 592} In 1991, Weidner

and colleagues used antiendothelial cell antibodies to identify tumor vasculature and showed that microvessel density was a useful prognostic marker for human breast cancer.⁸⁹ Microvessel density measures the relative intensity of angiogenesis in angiogenic clones from one tumor to another. In the past 10 years, microvessel density quantification has become a reproducible prognostic factor for risk of metastasis. The most plausible explanation for this correlation is that most human tumors contain angiogenic and nonangiogenic cells, both of which enter the circulation, but only the angiogenic cells can form detectable metastases.² Although there are a few reports in which microvessel density did not correlate with risk of metastasis or mortality, this could be due to technical or biologic causes. For example, if a primary tumor that was the source of a biopsy for quantification of microvessel density, generated a high level of circulating angiogenesis inhibitor(s), which suppressed growth of distant metastases, the prognostic value of this test could be negated. For a review of more than 50 publications of microvessel density quantified in more than 8,000 patients as a prognostic indicator of future metastasis or mortality, see Gasparini et al.^{561,558,593}

Although microvessel density is a useful prognostic marker of metastatic risk, it is not a good indicator of therapeutic efficacy for several reasons. In normal tissues, degree of vascularization and oxygen/nutrient demand are tightly coupled. In tumors, however, degree of vascularization and tumor growth are loosely coupled, or even uncoupled. This may be because in tumor cells, expression of angiogenic factors, such as VEGF, are no longer regulated by oxygen concentration. During tumor regression under antiangiogenic therapy, microvessel density may decrease if capillary dropout exceeds tumor cell dropout (autolysis), increase if tumor cell dropout exceeds capillary dropout, or remain the same if disappearance of capillaries and tumor cells parallel each other (Figure 11-16). Figure 11-16 shows osteosarcomas taken from mice treated with endostatin until there was more than a 50% inhibition of growth.²⁵² Despite the fact that this drug inhibited growth of both treated tumors depicted, the intensity of vascularization after treatment differed significantly between the tumors. Microvascular density was quantified over the entire histological section rather than over vascular hotspots, in order to avoid the effects of heterogeneity of vascularization in the tissue sample. Microvascular density dropped sharply in one treated tumor but rose slightly in the second, yet both tumors were equivalently reduced in size by the treatment relative to control. Thus, detection of a decrease in microvessel density during treatment with an angiogenesis inhibitor, suggests that the agent is active. However, the absence of a decrease in microvessel density does not indicate that the agent is ineffective.⁵⁵⁸ [↑ TOP](#)

Antiangiogenic activity of interferon-α: lessons for other angiogenesis inhibitors

Interferon-α has been widely used as an antiviral agent to treat chronic hepatitis, but also as a cytotoxic agent to treat certain leukemias and some bladder cancers.²⁵⁸ The first evidence that interferon-α had antiangiogenic activity was reported in 1980 when it was found to inhibit motility of

vascular endothelial cells in vitro in a dose-dependent and reversible manner, and subsequently found to inhibit angiogenesis in vivo. [244](#), [269](#), [270](#) Experimental studies in mice demonstrate that the antiangiogenic efficacy of interferon- α is optimal at low doses and declines at higher doses. [485a](#) New blood vessel growth in proliferating hemangiomas has been associated with increased expression of bFGF, and interferon- α has been shown to downregulate bFGF expression in human cancer cells. [239](#), [240](#), [243](#), [594](#), [595](#)

The first use of antiangiogenic therapy in a human was in 1988, when pulmonary hemangiomatosis in a 12-year old boy was successfully treated with low dose interferon- α therapy (3 million units/m²). [596](#), [597](#) Subsequently, infants with life-threatening or sight-threatening hemangiomas were successfully treated with the same low-dose interferon- α . [598](#), [599](#) Durable complete regressions have been achieved with low-dose interferon- α therapy in patients with solid tumors such as angioblastomas and giant cell tumors, where tumor angiogenesis was mediated mainly by bFGF. [239](#)–[241](#) Interferon- α at low doses has also been used to successfully treat hemangioendothelioma with or without metastases. [600](#)–[602](#) In a recent report, Kaban and colleagues treated recurrent high-grade giant cell tumors of the maxilla or mandible by simple surgical enucleation to spare nerves and teeth, and after 48 hours, patients received interferon- α , 3 million units/day subcutaneously for up to 8 months. [241](#), [242](#) All 8 of 8 patients had complete and durable regressions without recurrence for follow-up for up to 6 years. [TOP](#)

Clinical signs and symptoms in cancer patients that may be based on angiogenesis

Certain clinical signs and symptoms from tumor neovascularization are associated with specific tumor types. For example, retinoblastomas in the posterior eye induce iris neovascularization in the anterior chamber. Certain brain tumors induce angiogenesis in remote areas of the brain. Bone pain in metastatic prostate cancer may be related in part to neovascularization. A problem in the diagnosis of a primary bone tumor is that if the biopsy specimen contains only the neovascular response at the periphery of the tumor, it may be mistaken for granulation tissue or inflammation.

A variety of cancer syndromes, such as inappropriate hormonal activity, hypercoagulation, and cachexia, are secondary to the presence of biologically active peptides released into the circulation from vascularized tumors. Therefore, it might be predicted that an early therapeutic effect of antiangiogenic therapy would be increased appetite, weight gain, and disappearance of certain cancer syndromes. This early therapeutic effect would be most apparent with those angiogenesis inhibitors that had the least side effects. The angiogenesis induced by cervical cancer may be observed by colposcopy; the appearance of telangiectasia or "vascular spiders" in a mastectomy scar may herald local recurrence of tumor; color Doppler imaging can demonstrate neovascularization in breast cancer and other tumors; bladder carcinoma is detected by cystoscopy based, in part,

on its neovascularization; and mammography often reveals the vascularized rim of a breast tumor. ^{22, 445} In fact, a wide range of radiologic signs of cancer are based on "enhancement" of lesions by radiopaque dyes trapped in the neovasculature of a tumor. Moreover, in some tumors, large central areas cannot be penetrated by radiopaque dyes because of vascular compression, a situation that is unusual in prevascular tumors. [↑ TOP](#)

Future directions

There are currently so many clinical trials of angiogenesis inhibitors in the United States and other countries that space does not allow a summary of these trials. However, two recent reports validate the efficacy of antiangiogenic therapy for recurrent tumors when conventional therapy has failed. Kaban and colleagues report complete and durable regression of recurrent high grade giant cell tumors of the maxilla and mandible in adults and children by low-dose daily α -interferon for eight months, initiated 48 hours after simple enucleation surgery. ²⁴¹ Aiello and colleagues report the first case of treatment of an ocular hemangioblastoma with an angiogenesis inhibitor that blocks the receptor for VEGF. ²¹³ In this patient there was rapid and durable return of vision. The rapidity of return of eyesight was at the outset due to inhibition of increased permeability of the tumor by the antiVEGF regimen.

Need for surrogate markers of antiangiogenic efficacy

As translation of antiangiogenic therapy for cancer continues in clinical trials, there are several important challenges ahead. There is a need for surrogate markers of antiangiogenic efficacy. Maximum tolerated dose is not a useful indicator for antiangiogenic therapy. Administration of antiangiogenic therapy may be more analogous to insulin therapy than to chemotherapy. However, there is as yet no way to titrate dosing of angiogenesis inhibitors to the output of proangiogenic activity of the patient's tumor burden. Microvessel density, although useful as a prognostic indicator, is not as useful to determine efficacy of therapy. Quantification of proangiogenic factors such as VEGF, although correlative with prognosis in a few reports, may not measure all of the positive and negative regulators of angiogenesis generated in a tumor bed. Preliminary data indicates that quantification of circulating endothelial cells may become a promising surrogate marker of efficacy of antiangiogenic therapy, but much work remains to be done. Detection of novel circulating molecular markers of ongoing angiogenesis is a wide open opportunity for molecular biologists. [↑ TOP](#)

Combinations of angiogenesis inhibitors

Combinations of angiogenesis inhibitors will need to be tested in the future, as single angiogenesis inhibitors are approved for use in cancer. For example, it will be important to know whether bisphosphonates are synergistic with certain natural angiogenesis inhibitors such as angiostatin, endostatin, thrombospondin, or tumstatin. [↑ TOP](#)

Preventative antiangiogenic therapy

Preventive antiangiogenic therapy may also be possible in the future. Because antiangiogenic therapy is generally less toxic and less susceptible to induction of acquired drug resistance, it is possible that angiogenesis inhibitors could be used as prophylactic therapy in patients who have a high risk for cancer or for recurrence of cancer. An experimental study of spontaneous carcinogen-induced breast cancer in rats revealed that endostatin prevented the onset of breast cancer and also prolonged survival, compared with untreated controls. ⁴⁴⁹ Another example of preventive antiangiogenic therapy would be therapy guided by a molecular marker even before a tumor is visible. For example, in medullary carcinoma of the thyroid after surgical removal of the primary tumor, secondary tumors sometimes appear in the mediastinum and other sites. Because tumor recurrence can be preceded by rising serum calcitonin as early as 1 year or more before the recurrent tumor itself is detectable, it may in the future be feasible to give antiangiogenic therapy and follow the decline in calcitonin level analogous to the treatment of high grade giant cell tumors with low-dose interferon, where bFGF is the marker that is followed. ²³⁹ [⬆ TOP](#)

Antiangiogenic foods

It is also possible that in the future, foods that have high levels of antiangiogenic substances may be studied in clinical trials for prevention of cancer or its recurrence. Recently, there has been a summary of foods reported to contain antiangiogenic substances. ⁶⁰² [⬆ TOP](#)

Antiangiogenic gene therapy

Finally, in the long-term, antiangiogenic gene therapy may be useful, especially for the endogenous angiogenesis inhibitors that are proteins. For example, would it be possible to achieve slightly elevated levels of endostatin in the blood similar to the levels in patients with Down syndrome? The question could then be addressed as to whether this would benefit patients at high risk of new tumors such as women with the breast cancer gene, or with a high risk of tumor recurrence after treatment of the initial primary tumor. [⬆ TOP](#)

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